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Shh Signalling and the Specification of Neuronal Identity

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Presented for the PhD degree

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ABBREVIATIONS

aa	amino acids
AP	anterior-posterior
BCC	Basal cell carcinoma
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
Ci	Cubitus interruptus
CiR	Cubitus interruptus repressor form
CNS	Central nervous system
Cos2	Costal-2
DAPI	4'-6-Diamidino-2-phenylindole
Dhh	Desert hedgehog
Dpp	Decapentaplegic
D-V	dorsal-ventral
EDTA	Ethylenediaminotetraacetic
EnR	Engrailed repressor
ES	Embryonic stem cells
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FL	Forelimb
FP	Floor plate
Fu	Fused
g	grams
GBS-Luc	Gli Binding Sites Firefly-Luciferase reporter
Gli3A	Gli3 activator form
Gli3R	Gli3 repressor form

GSK3β	Glycogen synthase kinase 3β
HD	Homeodomain
Hh	Hedgehog
HH st	Stage according to Hamburger-Hamilton
HIP	Hedgehog Interacting Protein
HL	Hindlimb
hpt	Hours after electroporation
Ihh	Indian hedgehog
IκB	Inhibitor of κB
IL	Interlimb
IRES	Internal ribosomal entry site
l	litre
MB	Medulloblastoma
MED	Medium
mg	milligram
ml	millilitre
μl	microlitre
mM	millimolar
MNs	Motor neurons
NC	Notochord
NF-κB	Nuclear factor κB
ng	nanograms
NLS	Nuclear localization sequence
Nm	nanometre
nM	nanomolar
N-Shh	amino terminal peptide of processed Shh

nt	nucleotide
omb	*optomotor blind*
P	Probability
p	Progenitors
p0	Progenitors of V0 interneurons
p1	Progenitors of V1 interneurons
p2	Progenitors of V2 interneurons
p3	Progenitors of V3 interneurons
PCR	Polymerase chain reaction
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PKA	cyclic adenosine monophosphate (cAMP) dependent protein kinase
pMN	Progenitors of motor neurons
Ptc	Patched
PVF	Perivitelline fluid
RMS	Rhabdomyosarcoma
σ	standard deviation
sal	*spalt*
S.E.M.	standard error of the mean
Shh	Sonic hedgehog
Smo	Smoothened
spz	*spätzle*
som	somites
Su(fu)	Suppressor of fused
TGF-β	Transforming Growth Factor β type

TRIS	Tris[hydroxymethyl]aminomethane
TUNEL	TdT-mediated dUTP-biotin nick end labelling
V/cm	Volts/centimetre
v/v	volume/volume
w/v	weight/volume
Xbra	*Xenopus brachyury*
Xgsc	*Xenopus goosecoid*
ZF	Zinc fingers
ZPA	Zone of polarizing activity

ABSTRACT

During development, many signalling factors behave as morphogens, long-range signals eliciting different cellular responses according to their concentration. In ventral regions of the spinal cord, Shh is such a signal and controls the emergence, in precise spatial order, of distinct neuronal subtypes. The Gli family of transcription factors play a central role in this process, however, how the graded aspect of Shh signalling controls differential gene expression has been unclear. To address this question we have taken a gain of function approach in chick embryos. We have designed dominant inhibitory and dominant active versions of Gli proteins that are sufficient to block all Gli mediated transcription or mimic positive Gli activity, respectively. Analysis of the effect of these constructs when ectopically expressed in chick embryonic spinal cord indicates that blocking Gli mediated transcription prevents ventral neural tube patterning and the generation of the appropriate neuronal subtypes resulting in the dorsalisation of the ventral neural tube. Conversely, Gli dependent transcription is sufficient to mediate the full range of Shh responses in the neural tube. Moreover, the 2-3 fold changes in Shh concentration, which are necessary to switch between alternative neuronal subtypes *in vitro*, can be mimicked *in vivo* by similarly small changes in the level of Gli activity. This analysis also indicated that cells integrate the level of Shh signalling over time suggesting that signal duration in addition to signal strength is an important parameter controlling dorsal-ventral patterning. Together, these data indicate that graded Shh signalling is translated into a gradient of Gli activity without substantial signal amplification and that small changes in the level of Gli activity are sufficient to orchestrate the patterning of the ventral neural tube.

1. INTRODUCTION

1.1 SIGNALLING MOLECULES: MORPHOGENS

A central question in developmental biology is how "homogeneous" populations of cells diversify to generate organized tissues that consist of different cell types, accurately positioned to facilitate the function of an organ. A common theme during embryonic development is the deployment of molecular cues from the environment to direct the assembly of tissues. These signals provide positional information to the tissue being patterned, instructing cells to adopt particular cell fates according to their position within the tissue. Thus the positional identity and consequent fate of a cell is determined by the extracellular cues to which it is exposed. Some patterning cues function locally to control cell fate requiring contact between signalling and receiving cells, while others act on cells positioned many cell diameters away from their source. Of these long distance signals, some appear to have the additional feature of acting in a concentration dependent manner to control multiple cell fates. These have been termed morphogens and signals with these properties act by spreading from their source into surrounding tissue to form a gradient of activity. This gradient provides positional information to responding cells and controls multiple cellular outcomes in a concentration dependent manner.

Consequently, defining and understanding the molecular mechanisms of morphogens function is an important question in developmental biology. According to Gurdon and Bourillot (2001), in order to characterise a signalling molecule as a morphogen it should fulfil the following criteria. First, it should be present in the right place at the right time to provide positional information. Second, it should be released from a localized source to form a concentration gradient over a population of nearby and distant cells. Third, cells should respond to the signalling molecule in a concentration dependent way to generate multiple outcomes. Thus cells responding to the gradient should show two or

more qualitatively outputs such as the expression of different genes. Fourth, over- or under- provision of the signal should change gene expression or cell fates in the predicted directions. Thus an increase in signal such that all the cells experience elevated morphogen concentration should cause them to switch their response to a higher level. Likewise, under-provision should cause cells to change to a lower response. Finally, to be considered a morphogen its activity should be direct: wherever a cell is positioned in the concentration gradient it should respond directly to the morphogen, not indirectly through the mediation of other signalling molecules (Gurdon and Bourillot, 2001).

Few examples have been identified that meet all these criteria. Some of the most convincing and best studied examples of graded signal action in development are the patterning of the dorsal-ventral axis of *Drosophila* embryo, the patterning of mesoderm formation in *Xenopus* blastula and the patterning of the dorsal-ventral axis of vertebrate nervous system. I will briefly consider these examples below.

1.1.1 Dorsal: patterning along the dorsal-ventral axis of the *Drosophila* embryo

Patterning along the dorsal ventral axis of the *Drosophila* embryo is controlled by a nuclear gradient of the transcription factor Dorsal. Dorsal is homologous to mammalian NF-κB (Rushlow and Warrior, 1992). Like NF-κB, Dorsal activity is regulated at the level of subcellular localization (Rushlow and Warrior, 1992; Wasserman, 1993). An inhibitory protein, Cactus, which is related to mammalian IκB binds to Dorsal and the complex is retained in the cytoplasm. Upon activation by extracellular signals the complex dissociates and Dorsal translocates into the nucleus where it binds specific DNA target sites to regulate the expression of a set of genes (Wasserman, 1993).

In the precellular *Drosophila* embryo different concentrations of Dorsal in the nuclei specify different cell fates. Dorsal initiates the subdivision of the embryo into mesoderm, giving rise to muscles and connective tissue; neurogenic ectoderm, generating neural tissue; dorsal ectoderm, which gives rise to dorsal epidermis; and prospective amnioserosa a membrane in the dorsal side of the embryo that is eliminated later in embryonic development (Morisato and Anderson, 1995).

The graded distribution of Dorsal depends on establishment of the dorsal-ventral axis of the *Drosophila* embryo. This axis is initiated in the follicle cells surrounding the unfertilized egg in the ovary. The ventral pole of this axis is determined by the localized processing of a maternal protein, Spätzle (Spz). Spz is a signalling molecule secreted in an inactive state into the perivitelline fluid (PVF) that separates the oocyte and the follicle cells. The localized activation of a protease cascade in ventral regions of the PVF leads to the localized processing and activation of Spz ligand (LeMosy et al., 1999). Thus, the activated, proteolytically processed ligand is generated ventrally, spreads laterally and selectively activates the ubiquitous Toll receptor in ventral regions of the precellular embryo (Roth, 1994). Activation of Toll receptor triggers a signalling pathway which results in the re-localisation of the transcription factor Dorsal from the cytoplasm to the nucleus (Belvin and Anderson, 1996).

The exact mechanism responsible for the formation of Dorsal nuclear gradient along the dorsal ventral axis of the *Drosophila* embryo is unclear. It has been suggested that diffusion of the active form of Spz forms an extracellular ligand gradient which leads to a gradient of activated receptor Toll and Dorsal nuclear transport (Roth, 1993). There is also evidence that Spz-Toll complexes formed in ventral regions might be able to diffuse into lateral regions within the plasma membrane of the precellular embryo (Huang et al.,

1997). Regardless of mechanism, Dorsal nuclear transport appears to depend on the absolute number of its fully activated Toll receptors (Anderson et al., 1985; Huang et al., 1997). A large number of activated receptors lead to the complete transport of Dorsal from the cytoplasm to nuclei in ventral regions of precellular embryos (Stathopoulos and Levine, 2002a). In more lateral regions there are fewer activated Toll receptors and consequently lower levels of Dorsal enter the nuclei (Stathopoulos and Levine, 2002a). In dorsal regions of the embryo Spz and Toll receptors are inactive, therefore Dorsal remains cytoplasmic.

The extracellular Spz gradient is transduced intracellularly as a nuclear gradient of Dorsal and this patterns the dorsal-ventral axis of the *Drosophila* embryo. At different nuclear concentration thresholds Dorsal induces the expression of different target genes. *Twist* and *Snail* are activated when high levels of nuclear Dorsal protein are present, in a strip of 18-20 nuclei along the ventral side of the embryo that gives rise to the mesoderm (Kosman et al., 1991). Low levels of nuclear Dorsal, induce the expression of *rhomboid* in the neuroectoderm laterally (Bier et al., 1990). Other genes, for example the *Decapentaplegic (Dpp)*, which specifies amnioserosa, is repressed by Dorsal and is therefore only expressed dorsally where there is no nuclear Dorsal protein. How genes respond to different concentrations of Dorsal protein has been intensively investigated (Anderson et al., 1985; Stathopoulos and Levine, 2002a). The affinity of Dorsal binding sites in the regulatory regions appears critical in some genes. The proximal element of *Twist* enhancer, which drives expression in the 12-14 most ventral nuclei, contains two low affinity Dorsal binding sites. Conversion of these two sites into high affinity Dorsal binding sites results in broader expression in the 18-20 most ventral nuclei consistent with the idea that in most ventral regions where concentration of nuclear Dorsal is high, low affinity sites are sufficient to induce gene expression, whereas high affinity sites are

required for expression in more dorsal regions, where concentration of active Dorsal is lower (Jiang and Levine, 1993). In other genes the synergistic action of Dorsal and other transcription factors determine the domains of gene expression. For example Dorsal functions synergistically with Twist to activate and generate sharp boundaries of *Snail* expression (Ip et al., 1992b; Stathopoulos and Levine, 2002a). In addition inhibitory interactions with other transcription factors are involved. For example Snail protein represses the expression of certain genes (e.g. *rhomboid*) in ventral regions thus confines their expression laterally, to the neuroectoderm (Stathopoulos and Levine, 2002a).

1.1.2 Activin: patterning the mesoderm of the *Xenopus* blastula.

A second example of gradient signal is Activin and its function in patterning mesoderm of *Xenopus* blastula. Activin is a member of the transforming growth factor type β (TGF-β) superfamily and was the first factor characterized as a mesoderm inducer (Smith et al., 1990). Although Activin does not appear to be the endogenous mesoderm inducer the *in vitro* activity of Activin is an excellent model for morphogen action and is believed to simulate the action of endogenous *Xenopus* nodal proteins (Gurdon and Bourillot, 2001). Recently, this view was challenged by Piepenburg et al., (2004), their results suggesting that Activin is required for normal mesoderm formation in *Xenopus* embryos.

Exposure of animal cap explants (prospective ectoderm of *Xenopus* blastula) to low concentrations of Activin results in induction of lateral mesoderm marker *Xbrachyury (Xbra)*, while exposure to three fold higher concentration of Activin results in induction of dorsal mesoderm marker *Xgoosecoid (Xgsc)* (Gurdon et al., 1994). In support of this, when Activin loaded beads are placed in a spherical cell aggregate of animal cap cells or in two face to face animal caps a wave of *Xbra* gene expression spreads out radially from the activin beads and this is followed by the expression of *Xgsc* in more centrally located

cells (Gurdon et al., 1994). Thus, *in vitro*, Activin functions in a concentration dependent manner and at different concentration thresholds induces different mesodermal markers.

A single receptor complex mediates both low and high level Activin responses. Moreover, careful quantitation indicated that cells sense ligand concentration by the absolute number of occupied receptors per cell; 100 and 300 molecules of liganded receptors corresponding to 2% and 6% of the total receptors induce *Xbra* and *Xgsc*, respectively (Dyson and Gurdon, 1998). This indicates that three fold differences in Activin level that switch between low and high responses are mediated by a three fold difference in number of liganded/activated receptors (Dyson and Gurdon, 1998).

Activin binding induces the heterodimerization of the activin serine-threonine kinase receptors. This results in the phosphorylation and activation of Smad2 protein which enters the nucleus and acts as transcriptional activator. Three fold differences in active Smad2 concentration mimic the effect of three fold difference in extracellular Activin concentration, causing a switch in *Xbra/Xgsc* gene expression (Shimizu and Gurdon, 1999). Together these data suggest that morphogen concentration is transmitted from outside the cell to the nucleus by a linear pathway via activation of Smads without substantial signal amplification.

The last example of morphogen is Sonic hedgehog (Shh), which functions as a long range graded signal to pattern the developing spinal cord along the dorsal ventral axis (Briscoe et al., 2001; Briscoe and Ericson, 2001; Ericson et al., 1997a; Jessell, 2000; Roelink et al., 1995; Wijgerde et al., 2002). This will be described in detail below.

1.1.3 Time as an important parameter for tissue patterning

The definition of a morphogen indicates that it must act directly on target cells and a cell's response to the morphogen depends on the concentration to which it is exposed. This has led to concepts of spatial patterning. Additionally, however, the duration of exposure to a signal may play an important role. Indeed, Pages and Kerridge (2000) proposed an alternative model for morhogen action, which they termed the "sequential cell context" model. In this model signal duration rather than spatial arrangement is the important determinant of cellular responses to a morphogen. They suggested that initial signalling activates an early set of target genes that change the cell context in those cells where signalling first occurs. This change in cell context acts synergistically with continued signalling to allow the expression of a later set of target genes. Consistent with this, many known early target genes encode transcription factors that are required for patterning and it is possible that their expression modifies the cell context and affects later signalling responses. A similar model, termed the "self enabling mechanism" was proposed by Kang et al. (2003). This study provided evidence that TGFβ signalling mediator, Smad3, activates expression of stress response factor ATF3. Once induced ATF3 is recruited by Smad3 to *Id1* promoter. Recruitment of ATF3 enables Smad3 to repress expression of Id1 in epithelial cells (Kang et al., 2003). Thus a Smad3 mediated primary gene response, the induction of ATF3, enables Smad3 to participate in a secondary gene response, i.e. repression of Id1, which Kang et al. (2003) described as a "self enabling" mechanism.

Among the examples of morphogen function that Pages & Kerridge proposed operate via a sequential cell context model are the *Decapentaplegic (Dpp)* signal in the *Drosophila* imaginal disc and activin signal in *Xenopus* blastula. *Dpp* is expressed in a group of cells along the anterior-posterior boundary of the wing imaginal disc. There is an

absolute requirement for Dpp for the expression of two target genes, *optomotor blind (omb)* and *spalt (sal);* Omb is expressed in cells further from the source of Dpp than Sal. Thus low Dpp levels induce *omb*, while high Dpp levels induce *sal* and *omb* (Lecuit et al., 1996; Nellen et al., 1996). Ectopic expression of Dpp in clones of cells results in the expression of *omb* in a large domain and later *sal* is induced in a nested circle within the *omb* region (Lecuit et al., 1996; Nellen et al., 1996). Dpp expression levels have been varied by using strong or weak promoters (Lecuit and Cohen, 1998; Nellen et al., 1996). Weak expression of Dpp induces *omb* before *sal* (24h and 72h respectively), with *omb* detectable in more cells than *sal* (Nellen et al., 1996). Strong expression of Dpp induces almost simultaneous expression of both target genes (Lecuit and Cohen, 1998). These data could be interpreted as indicating that high concentration of Dpp induces both *sal* and *omb*, while low concentration will only induce *omb* (Lecuit and Cohen, 1998; Nellen et al., 1996). According to the sequential cell context model the same data could be indicating that low Dpp concentration induces *omb*. Then low Dpp acting synergistically with Omb (or other early targets) induces *sal*.

Pages and Kerridge also propose an alternative explanation to the dose dependent activity of Activin described earlier. They suggest that at low doses of Activin only *Xbra* is induced because not enough time has elapsed to get the secondary response. At high concentrations primary and secondary responses occur (induction of *Xbra* and *Xgsc* respectively) as the whole process is accelerated.

More evidence about the importance of duration of exposure to the signal on patterning comes from the role of Shh signalling in vertebrate limb development and the determination of digit identity. Shh is expressed by cells in the posterior of the developing limb in the region called zone of polarizing activity (ZPA) and either

directly, or by inducing another secreted factor, Shh patterns the developing limb along the anterior-posterior (AP) axis (Helms et al., 1994; Riddle et al., 1993). Grafting of ZPA cells or beads loaded with Shh in the anterior of the limb bud results in the formation of extra digits with reverse AP polarity and this was described as a concentration dependent response; the more ZPA cells were grafted or the higher the concentration of Shh in the beads, the more posterior identity the ectopic digits assumed (Tickle, 1981; Yang et al., 1997). In addition to the concentration, duration of exposure to Shh signal seemed to be important for determination of ectopic digit identity; shorter exposure of cells to Shh resulted in formation of ectopic digits with anterior identity while longer exposure resulted in ectopic digits with posterior identity (Smith, 1980; Yang et al., 1997). Recent studies (Ahn and Joyner, 2004; Harfe et al., 2004) suggest that for the specification of the two most posterior digits, concentration of Shh is not important since both descend from Shh expressing cells, however cells that will generate the most posterior digit (fifth) express Shh for longer time compared to those that will form the forth digit. These data suggest that in addition to a spatial gradient of Shh concentration a temporal Shh gradient is important for digit specification.

Taken together these data suggest that in addition to strength, duration of exposure to a signal could affect cellular responses thus it is important to consider time as well as concentration as parameters of morphogen activity. Indeed the two parameters, time and strength may be directly related. It is possible to reconcile the concentration of signal and the duration of signal models with a model that proposes signal accumulation; increasing concentration or increasing duration of signalling could result in activation of the pathway and accumulation of an intracellular correlate of signal strength such as activation of a transcription factor. In this view concentration of signal could be integrated over time and this integration determines the responses.

1.2 FORMATION OF THE SPINAL CORD

The vertebrate central nervous system (CNS) consists of the brain and spinal cord. Essential for its function is the generation of numerous distinct neuronal cell types in appropriate numbers and at precise positions. The CNS emerges from the neural plate, a sheet of multipotent neuroepithelial cells flanked by epidermal ectoderm and underlayed by mesodermal tissue, the notochord in the midline and the paraxial mesoderm laterally (Diez del Corral and Storey, 2004). As development proceeds, neural plate folds to form the neural tube. Under the influence of signals from the neighbouring tissues, the multipotent proliferating cells adopt a pattern of regional identity; they become progenitors devoted to generate distinct neuronal subtypes. Subsequently, these different progenitor populations begin to generate post-mitotic neurons that acquire characteristic identities and the distinct functional properties from which mature circuits are assembled.

Here I will focus on the patterning of the spinal cord. This represents the most experimentally tractable region of the CNS and has long been used to investigate mechanisms of neural development. The neurons of the mature spinal cord serve two major functions (Jessell, 2000). They relay sensory input to higher centers in the brain and they coordinate motor output. The neuronal circuits that mediate these functions are to a large extent anatomically segregated. Neurons involved in the processing of sensory input are located in the dorsal half of the spinal cord, whereas neurons involved in motor output reside in the ventral half of the spinal cord. This spatial segregation results from the generation of distinct neuronal types from distinct regions along the dorsal-ventral axis of the neural tube.

1.2.1 Patterning Signals

Signals emanating from adjacent tissues provide neural tube cells with positional information and induce the appropriate cell identities along the dorsal ventral axis. A series of studies have focused attention on the notochord (NC), the rod of axial mesodermal cells that underlies the neural tube, and the floor plate (FP), a group of specialized glial cells occupying the ventral midline of the neural tube (review by (Placzek et al., 1991). In the 1980s it was suggested, based on NC transplantation experiments in chick that NC can influence neural cell differentiation (van Straaten et al., 1985a; van Straaten et al., 1985b). Subsequently more detailed analyses of NC transplantation and ex vivo experiments, facilitated by the identification of markers characteristic of different neuronal types, showed that differentiation of neural cells is controlled in part by their position with respect to notochord NC and FP cells (Placzek et al., 1991; Yamada et al., 1991) and a diffusible signal secreted from NC and FP mediated this patterning effect (Yamada et al., 1993). This diffusible signal was subsequently identified as the amino-terminal peptide of processed Sonic Hedgehog (Shh), N-Shh (Marti et al., 1995; Roelink et al., 1995), a homologue of *Drosophila* Hedgehog (Echelard et al., 1993). Since, intense research in this field has confirmed the function of Shh in specifying progenitor cell fate and neuronal subtype identity (Briscoe and Ericson, 2001; Ingham and McMahon, 2001; Jessell, 2000). Indeed Shh appears to act as a long-range graded signal in a concentration dependent manner (Fig. 1) (Briscoe et al., 2001; Ericson et al., 1997a; Gritli-Linde et al., 2001; Hynes et al., 2000; Lewis et al., 2001; Wijgerde et al., 2002).

Other signalling molecules have also been implicated in playing important roles in patterning the ventral neural tube independent of Shh. Retinoids derived from somitic

and presomitic mesoderm are important for the specification and differentiation of motor neurons and interneurons in the ventral and intermediate spinal cord (Diez del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 1999). Also Fibroblast Growth Factors (FGFs) secreted by the paraxial mesoderm and Hensen's node are essential for the formation of neural tissue with caudal character and affect the expression of progenitor markers along the dorsal ventral axis of the spinal cord (reviewed by Diez del Corral and Storey, 2004). Other secreted factors that influence patterning in this tissue include the Wingless/Int-related (Wnt) and bone morphogenetic protein (BMP) signals that emanate from the dorsal neural tube and overlaying ectoderm (Lee and Jessell, 1999).

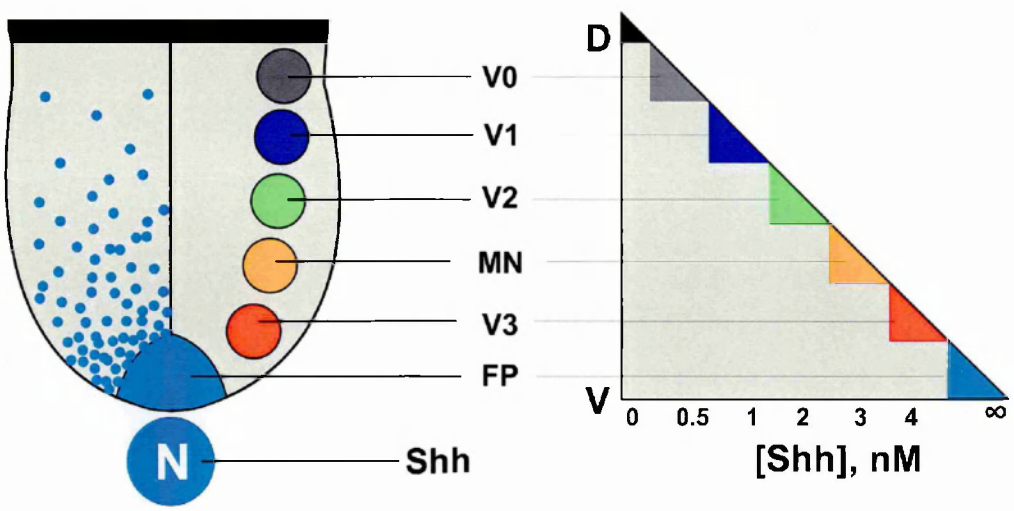


Figure 1. Shh is secreted from notochord and floor plate cells and at different concentration thresholds induces different neuronal subtypes (Briscoe and Ericson, 2001).

1.2.2 Interpretation of graded Shh signal

Shh signalling acts by regulating the spatial pattern of expression of transcription factors that include homeodomain proteins of the Nkx, Pax, Dbx, and Irx families and the bHLH protein Olig2 (Briscoe et al., 2000; Ericson et al., 1997b; Muhr et al., 2001; Novitch et al., 2001; Pierani et al., 1999; Vallstedt et al., 2001). These transcription factors are subdivided into two groups, termed class I and II proteins, based on their mode of

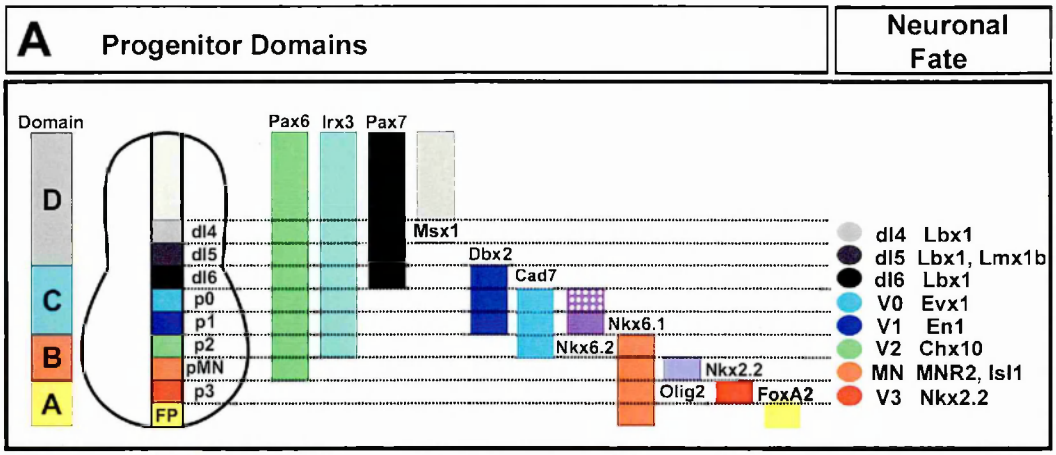
regulation by Shh (Briscoe et al., 2000). Class I proteins appear to be constitutively expressed by neural progenitor cells, and their expression is repressed by Shh signalling, whereas neural expression of the class II proteins is dependent on Shh signalling (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997a; Pabst et al., 2000; Qiu et al., 1998; Vallstedt et al., 2001). Manipulation of Shh signalling changes the pattern of class I and class II gene expression in predictable fashion (e.g. Briscoe et al., 2001).

The differential transcriptional responses to graded Shh signalling of class I and class II proteins suggest how the pattern of progenitor domains is initially established, but it is difficult to account for the strikingly sharp boundaries between domains of expression solely by this mechanism. Indeed gain and loss of function studies suggest that cross-repressive interactions between complementary pairs of class I and class II which share the same progenitor domain boundary is important to refine domains of gene expression. For example, Pax6 (class I) and Nkx2.2 (class II) share a progenitor domain boundary. Loss of Pax6 function results in the dorsal expansion of Nkx2.2 expression and Pax6 is sufficient to repress Nkx2.2 expression (Briscoe et al., 2000; Ericson et al., 1997b). Conversely, Nkx2.2 is sufficient to repress Pax6 expression *in vivo* (Briscoe et al., 2000). In a similar way, Dbx2 (class I) and Nkx6.1 (class II), Dbx1 (class I) and Nkx6.2 (class II) as well as Irx3 (class I) and Olig2 (class II) are expressed in mutually exclusive progenitor domains and a cross-repressive relationship is evident within each pair of class I and class II proteins (Briscoe et al., 2000; Novitch et al., 2001; Sander et al., 2000; Vallstedt et al., 2001). A complementary class II protein for the class I Pax7 has not been identified.

The profile of homeodomain proteins expressed by progenitor cells act to specify the identity of the neurons derived from each progenitor domain. The three most ventral

progenitor (p) domains, p2, pMN and p3 generate V2 neurons, motor neurons (MNs) and V3 neurons, respectively. The combinatorial activities of three homeodomain proteins Nkx6.1, Nkx2.2, Irx3 and bHLH protein Olig2 control the position of generation of these neuronal subtypes. Nkx6.1, in the absence of Irx3 induces MN generation (Briscoe et al., 2000) and loss of Nkx6.1 activity results in decreased production of MNs (Sander et al., 2000; Vallstedt et al., 2001). Although Nkx6.1 is also expressed by progenitors that generate V2 and V3 neurons, MN induction is restricted in these domains by Irx3 and Nkx2.2; Irx3 and Nkx2.2 repress Olig2 restricting its expression to the pMN domain (Novitch et al., 2001). Progenitors co-expressing Nkx6.1 and Nkx2.2 generate V3 neurons (Briscoe et al., 2000; Briscoe et al., 1999).

To summarise, the model that outlines how graded Shh signal controls the pattern and identity of neurons in the ventral neural tube, proposed by Briscoe et al., 2000 (Briscoe and Ericson, 2001; Briscoe et al., 2000), can be divided in three steps. First, graded Shh signalling initiates dorsal-ventral restrictions in the expression domains of class I and class II proteins within the ventral neural tube. Class I proteins are repressed by Shh signal and class II proteins require Shh signalling. Individual class I and class II proteins have different concentration requirements for repression or activation. Second, cross-repressive interactions between class I and class II proteins that share a common progenitor domain boundary refine and maintain progenitor domains. Finally, the profile of expression of class I and class II proteins within an individual progenitor domain controls neuronal fate. This three step model describes how graded Shh signalling organises ventral neural tube patterning but leaves open the question of how the expression of class I and class II proteins are initially regulated by Shh signalling.



B

Drosophila	Vertebrates
Hh	Shh, Ihh, Dhh
ptc	Ptc1, Ptc2
smo	Smo
Cos2	Not identified
Fu	Fu
Su(fu)	Su(fu)
ci	Gli1, Gli2, Gli3

Figure 2. (A) Diagram of class I and class II protein expression and the relationship between progenitor proteins and neuronal subtype identity.

(B) Components of Hh signalling pathway in *Drosophila* and vertebrates.

1.2.3 Hedgehog (Hh) signalling pathway

The molecular mechanisms of Hh signalling are best understood in *Drosophila*. Activity of the pathway is triggered by binding of Hh ligand to Patched (Ptc) (Fuse et al., 1999; Marigo et al., 1996a; Stone et al., 1996), a transmembrane protein that in the absence of Hh acts catalytically to suppress activity of another transmembrane protein Smoothened (Smo) (Taipale et al., 2002). Initially it was thought that Ptc exerted a direct inhibitory interaction upon Smo which was relieved by binding of Hh to Ptc, possibly through a conformational change (Murone et al., 1999; Stone et al., 1996). Recent findings suggest an alterative possibility. Ptc and Smo do not seem to co-localize in the cell membrane in the absence of Hh signalling (Zhu et al., 2003) thus physical association does not appear

to be needed for Ptc to suppress Smo activity. In addition, upon ligand binding the signalling complex including the bound Hh is internalized into the cell. Subsequently, Ptc and Shh enter lysosomes for degradation, while Smo seems to be stabilized by phosphorylations and recycled to the cell surface (Incardona et al., 2002; Zhu et al., 2003). Zhu et al also suggest that the normal function of Ptc (in the absence of Hh) is to confine Smo to internal cell locations. Inactivation of Ptc by binding to Hh permits activation of Smo, which in turn results in activation of the transcription factor Cubitus interruptus (Ci).

Ci is critical to Hh mediated control of gene expression (Ingham, 1998; Ingham and McMahon, 2001; Methot and Basler, 2001). In the absence of Hh signalling, Ci is proteolytically processed into a truncated repressor form that inhibits Hh target genes (Aza-Blanc et al., 1997; Robbins et al., 1997). Hh signalling inhibits the processing of Ci, converting Ci into a transcriptional activator (Jia et al., 2002; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998; Price and Kalderon, 2002). Although the inhibition of cleavage is an essential step in the generation of a fully active Ci protein, Hh signalling also potentiates the transcriptional activity of Ci, possibly through a mechanism that increases its nuclear accumulation (Chen et al., 1998; Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000).

The repressor form (CiR) consists of an N terminal proteolytic fragment containing the zinc finger DNA binding domain and the repressor domain, but misses the C terminal activator domain. Formation of CiR requires phosphorylation of specific serine-threonine residues by cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) (Chen et al., 1998). PKA prosphorylation triggers further phosphorylation by glycogen synthase kinase 3β (GSK3β) and a member of the CK1 family of kinases (Jia et al.,

2002; Lum et al., 2003a; Price and Kalderon, 2002). The phosphorylated form of Ci appears to be a substrate for proteolytic processing that requires the function of the proteasome and of Slimb, an E3 ubiquitin ligase component (Jiang and Struhl, 1998; Theodosiou et al., 1998).

CiR formation appears to be regulated by interaction of Smo with Costal-2 (Cos2), a kinesin-like protein that stably associates with Ci and is required for Ci processing (Lum et al., 2003b; Robbins et al., 1997; Sisson et al., 1997). When cells are stimulated by Hh, Smo protein is stabilized (Lum et al., 2003b) and recruits Cos2 via sequences on its cytoplasmic tail (Jia et al., 2003; Lum et al., 2003b). This results in the loss of CiR, accumulation of uncleaved Ci and activation of the Hh target gene *Dpp* (Jia et al., 2003). *Dpp* is activated in the absence of CiR (Jia et al., 2003) suggesting that derepression is sufficient to activate some target genes. Loss of CiR alone, however, is insufficient to activate the full range of Hh responses because expression of *ptc* and other target genes is not induced under these conditions (Jia et al., 2003). These data indicate that derepression is not sufficient to activate all Hh target genes. Instead transcriptional activation is required for full Hh response.

Cos2 restricts Ci activator not only by promoting cleavage but in addition by anchoring Ci in the cytoplasm (Chen et al., 1999; Methot and Basler, 2000), a function also abolished upon exposure to Hh signal and the subsequent recruitment of Cos2 by Smo (Jia et al., 2003). Moreover Cos2 has positive input into the Hh pathway as it is necessary for the stabilization of serine-threonine kinase Fused (Fu) which inactivates Supressor of fused, Su(fu), a negative regulator of Hh/Ci (Lum et al., 2003b). Su(fu) physically interacts with Ci (Lum et al., 2003b) and appears to inhibit Ci activity by promoting the cytoplasmic localization of Ci (Methot and Basler, 2000). In addition

Su(fu) may also inhibit Ci function in the nucleus since it has been shown that Su(fu) translocates into the nucleus, has the ability to form a DNA binding complex with Gli proteins, the vertebrate homologues of Ci, and recruits to promoters with Gli binding element the SAP18-mSin3 histone de-acetylase co-repressor complex (Cheng and Bishop, 2002; Zhang et al., 1997).

Differential degradation has been suggested as an additional mechanism for regulation of Ci function in the eye imaginal disc. Degradation in the posterior of the eye disc depends on different factors from the anterior and results in complete degradation of Ci as opposed to selective removal of the C-terminal half of Ci (Ou et al., 2002).

1.2.4 Shh pathway

The Hh pathway in vertebrates parallels to a great extent that in *Drosophila* (Fig. 2B), although some of the components of the pathway are duplicated, and some do not have identified *Drosophila* homologues.

Three homologues of *Hh* have been identified in the mouse: *Desert hedgehog (Dhh), Indian hedgehog (Ihh)* and *Sonic hedgehog (Shh)* (Echelard et al., 1993). Their expression patterns are largely distinct and they seem to have different roles: Shh establishes the dorsal-ventral axis in the CNS, anterior-posterior axis in the developing limb and is important for development of somites, lungs and other organs; Ihh is involved in chondrocyte development and Dhh plays a role specifically in germ cell development reviewed by (Ingham and McMahon, 2001). Targeted disruption of *Shh* causes, among other defects, cyclopia and absence of ventral cell types in the neural tube (Chiang et al., 1996), confirming it has an important role in patterning the ventral nervous system.

Two *Ptc* genes have been identified in mammals. Loss of function in the first of these to be identified, *Ptc1*, leads to constitutive activation of Hh target genes, transforming most of, if not all, the neural plate into floor plate (Goodrich et al., 1997) and suggesting that, as is the case in *Drosophila*, Ptc1 functions to prevent activation of the pathway in the absence of Hh. Ptc2 also binds all mammalian Hh proteins but whether it plays a role in Hh signal transduction it is not clear (Carpenter et al., 1998). *Smo* is represented by a single mammalian homologue (Akiyama et al., 1997). Ectopic expression of an oncogenic form of Smo, SmoM2, results in the cell autonomous activation of Hh targets in the neural tube (Hynes et al., 2000). In addition *Smo* null mutant neural progenitor cells lose the ability to transduce Hh signal and do not acquire ventral identities (Wijgerde et al., 2002) consistent with Smo playing a positive role in the transduction of Hh signal.

Regarding the cytoplasmic components of mammalian Hh pathway, an orthologue of *Cos2* has not been identified. Fu and Su(fu) have been proposed to physically interact with and regulate subcellular localization of Gli proteins, the transcriptional effectors of the pathway (Murone et al., 2000). Su(fu) is capable of interacting with Gli proteins bound to DNA (Pearse et al., 1999) and also interacts with a histone de-acetylase complex suggesting that, in addition to possible role in regulating subcellular localization of Gli proteins, Su(fu) could affect transcriptional activity by recruiting chromatin modulating factors to the Gli binding DNA sites (Cheng and Bishop, 2002). *In vivo* roles of mammalian Fu and Su(fu) have not been reported. Loss of function analysis in zebrafish using morpholinos suggests that Fu functions as a mild activator, while Su(fu) functions as a mild repressor of Hh pathway (Wolff et al., 2003). Recent studies of *iguana* mutants in zebrafish revealed a novel component of Hh pathway, Dzip. Dzip

seems to function as an essential, permissive factor of Gli function, possibly by regulating nuclear levels of Gli proteins (Sekimizu et al., 2004; Vokes and McMahon, 2004; Wolff et al., 2004).

Several genes have been identified through genetic screens as components of the vertebrate Hh pathway that have either no identified *Drosophila* homologue or do not have known functions in Hh signalling in *Drosophila* (Bulgakov et al., 2004; Nybakken and Perrimon, 2002). These additional components function at different steps along the Hh transduction pathway. One of them, relevant to this study is Hedgehog Interacting Protein (HIP), a membrane bound glycoprotein, upregulated in response to Hh signal, that binds Shh and functions as a negative regulator of Shh signalling (Chuang and McMahon, 1999).

1.2.5 Glis

In vertebrates, three homologs of *Ci: Gli1, Gli2* and *Gli3* (Hui et al., 1994; Marigo et al., 1996a; Marigo et al., 1996b; Ruppert et al., 1988), have been identified and are expressed in the neural tube (Brewster et al., 1998; Ingham and McMahon, 2001; Lee et al., 1997; Sasaki et al., 1997). Gli2 and Gli3 proteins share similar domain organization as Ci including five C2H2 type zinc fingers that form a DNA binding motif flanked at the amino half of the protein by repressor of transcription domain and at the carboxy half of the protein with an activator domain (Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1999). Gli1 also has five C2H2 zinc fingers DNA binding motif and an activator domain at the carboxy half of the protein but it does not contain repressor sequence (Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1999). The situation in vertebrates is further complicated by the fact that the genes encoding Gli1 and Gli3 are transcriptional targets of Hh signalling. *Gli1* is activated and *Gli3* is repressed in response to Hh signals

(Lee et al., 1997; Marigo et al., 1996b). *Gli1* is expressed in the ventral neural tube and its expression depends on Hh signalling (Bai et al., 2002), *Gli2* and *Gli3* are expressed in the neural tissue before neural tube closure (Hui et al., 1994; Lee et al., 1997). As development proceeds *Gli2* expression remains uniform, while *Gli3* becomes restricted to dorsal and intermediate neural tube (Lee et al., 1997).

Expression of Gli proteins in an heterologous system, the *Drosophila* imaginal disc, suggested that Gli1 and Gli2 function as Hh dependent activators of Hh target genes, while Gli2 and Gli3 but not Gli1 function as repressors in the absence of Hh (Aza-Blanc et al., 2000; von Mering and Basler, 1999).

Analyses of truncated Gli proteins revealed their potential both to activate and repress transcription from synthetic promoters containing Gli binding sequences, except Gli1, which does not have a repressor domain and functions only as an activator (Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1999). Gli2 and Gli3 (but not Gli1) can be proteolytically processed in a manner similar to Ci to form transcriptional repressors (Aza-Blanc et al., 2000; Dai et al., 1999; Wang et al., 2000).

Gli1, 2 and 3 bind to Ci related consensus sequences that are present in several Shh responsive genes (Agren et al., 2004; Dai et al., 1999; Sasaki et al., 1997; Sasaki et al., 1999; Yoon et al., 2002). Gli binding sites have been identified in the regulatory/promoter sequences of *FoxA2, Ptc1* and *Gli1* suggesting these genes are direct targets of Shh signalling and their expression can be regulated by Gli transcription factors (Agren et al., 2004; Dai et al., 1999; Sasaki et al., 1997). Interestingly, Zic proteins, which are zinc finger transcription factors, can bind *in vitro* the same sequences as Gli proteins (Mizugishi et al., 2001). *Zic1-3* are expressed in the dorsal neural tube

and have been proposed to be important for neural crest development (Brewster et al., 1998; Nagai et al., 1997).

1.2.6 Shh signalling in the ventral neural tube

Shh signalling is important for ventral neural tube patterning. Blockade of Shh signalling with Shh specific antibodies blocked induction of FP by the NC and induction of MNs by the NC and FP (Ericson et al., 1996). Consistent with this, *Shh* null embryos display dramatic patterning defects resulting in the loss of most ventral neuronal subtypes (Chiang et al., 1996; Pierani et al., 1999). Furthermore, Shh signalling functions in a graded manner. *In vitro* experiments analysing intermediate neural tube explants exposed to incremental concentrations of Shh showed that at different concentration thresholds Shh induces different neuronal identities (Ericson et al., 1997a; Roelink et al., 1995). For example, 2nM Shh induced MNs while the minimum concentration for induction of V3 interneurons was 3nM Shh (Ericson et al., 1997a). In addition, there is a correlation between the concentration of Shh required to induce a neuronal type *in vitro* and the distance from the ventral midline that it is generated *in vivo* (Ericson et al., 1997a; Roelink et al., 1995); Induction of V3 interneurons, which are generated ventrally next to the floor plate cells, requires higher concentration of Shh compared to V2 interneurons which are generated at a distance from the ventral midline.

Studies in which Shh signal was blocked in a mosaic, cell autonomous way indicated that Shh also acts at long range (Briscoe et al., 2001; Wijgerde et al., 2002). Overexpression of $Ptc1^{\Delta loop2}$, a deletion of Ptc1 which lacks the capacity to bind Shh but retains the ability to inhibit Smo or a downstream Smo effector in the presence of Shh, blocked Shh responses in electroporated chick neural tube cells positioned at a distance from the floor plate (Briscoe et al., 2001). Similar results obtained using ES cells lacking

Smo. In chimeric mouse embryos containing a mixture of wild type and $Smo^{-/-}$ cells, $Smo^{-/-}$ cells were unresponsive to Shh signal and did not express markers characteristic of the ventral neural tube (Wijgerde et al., 2002).

Taken together these findings indicate that Shh signalling exhibits the characteristic features of a morphogen: Shh functions in a graded manner, directly on cells positioned at a distance from the signal source to control dorsal-ventral patterning and the specification of cell fate. How the signal transduction pathway operates to mediate graded Shh signalling remains to be elucidated.

Embryos lacking Shh signalling display dramatic patterning defects resulting in the loss of most ventral neuronal subtypes (Chiang et al., 1996; Pierani et al., 1999) however the loss of individual *Gli* genes has relatively minor defects with MNs and V2 neurons being generated in each of the *Gli* null mutant embryos (Ding et al., 1998; Litingtung and Chiang, 2000; Matise et al., 1998; Park et al., 2000). In *Gli1* null mutant embryos dorsal-ventral patterning of the spinal cord is not affected (Matise et al., 1998; Park et al., 2000) while embryos lacking *Gli2* have defects in the most ventral regions of the neural tube, with defects in the generation of floor plate and V3 neurons (Ding et al., 1998; Matise et al., 1998; Park et al., 2000). These defects can be rescued by replacing *Gli2* with *Gli1*, leading to the suggestion that only activator function of Gli2 is required for normal patterning of the neural tube (Bai and Joyner, 2001). Interestingly in zebrafish embryos downregulation of Gli1 or Gli2 with morpholino oligonucleotides results in different phenotypes from $Gli1^{-/-}$ or $Gli2^{-/-}$ mouse embryos. Blocking expression of Gli1 protein results in defects in activation of Shh target genes (e.g. lack of Nkx2.2 expression), while blocking Gli2 results in only minor Hh signalling defects (Karlstrom et al., 2003).

Gain of function experiments suggest that Shh signalling acts to repress an inhibitory activity of Gli3 (Aza-Blanc et al., 2000; Lee et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1999; von Mering and Basler, 1999). Consistent with this, in mouse embryos lacking *Gli3*, there is a dorsal expansion of progenitor domains in the intermediate spinal cord, at the expense of more dorsal progenitors (Persson et al., 2002). These defects are rescued in embryos carrying a targeted allele of *Gli3* that encodes solely a repressor variant equivalent to proteolytically processed Gli3 (Bose et al., 2002; Persson et al., 2002). Moreover, in mice lacking both *Shh* and *Gli3* or *Smo* and *Gli3* the pattern of progenitor domains and neuronal subtype identity is, to a large extent, restored (Litingtung and Chiang, 2000; Persson et al., 2002) and development of motor neurons (MNs) and ventral interneurons is rescued (Litingtung and Chiang, 2000) substantiating the idea that ventral patterning can proceed via a mechanism independent, or parallel, to graded Shh signalling.

Recently *Gli2;Gli3* double mutant mouse embryos were analysed (Bai et al., 2004; Lei et al., 2004). They lack all Gli function as *Gli1* is not transcribed. In these embryos floor plate and V3 interneurons are missing as it is observed in *Gli2* mutant embryos. Motor neurons and V0-V2 interneurons are generated although they are intermingled instead of being spatially segregated, suggesting that Gli activity is not required for the generation of these neuronal types but is required for their correct positioning. Moreover, altered numbers of neuronal subtypes are generated in the absence of Gli activity, fewer motor neurons and V2 neurons are formed while the numbers of V1 and V0 neurons increase (Lei et al., 2004). Gli activity is possibly also important for motor neuron differentiation as in *Gli2;Gli3* double mutant embryos there are more motor neuron progenitors and less differentiated neurons compared to wild type. (Bai et al., 2004; Lei et al., 2004). These observations are consistent with the idea that Gli function is not required for the

generation of MNs and V2-V0 neurons, but it is required for their correct positioning and number.

1.2.7 Hh/Shh signalling, proliferation and cancer

In addition to the patterning role, Hh signalling has been associated with the regulation of cell growth and proliferation during different aspects of development. During *Drosophila* eye development Hh signalling has been suggested to promote transcription of *Cyclin D* and *Cyclin E*, two principal regulators of the cell cycle (Duman-Scheel et al., 2002). *Cyclin E* was suggested to be a direct target of Hh signalling since Ci can bind the *Cyclin E* promoter (Duman-Scheel et al., 2002). Furthermore, in studies of cerebellum development Shh signalling has been suggested to promote proliferation of granule neuron precursors by inducing directly the expression of the proto-oncogene *Nmyc* (Kenney et al., 2003). Blockade of the mitogenic effect of Shh signalling activation can be achieved by treating proliferating cells with cyclopamine *in vitro* and *in vivo* (Berman et al., 2002). Cyclopamine is a plant alkaloid which inhibits Shh signalling by directly interacting with Smo (Chen et al., 2002) and is a teratogen causing significant defects in the developing nervous system (Incardona et al., 1998).

Furthermore, Ptc1 has been proposed to prevent proliferation by directly interacting with Cyclin B1 and blocking its nuclear localisation, a function that can be relieved in the presence of Shh (Barnes et al., 2001). In addition to the regulation of proliferation Ptc1 has been proposed to regulate apoptosis in neuroepithelial cells (Thibert et al., 2003). Thibert et al suggested that Ptc1 induces apoptotic cell death unless its ligand is present to block the signal. The overgrowth observed in the *Ptc1*$^{-/-}$ embryos (Goodrich et al., 1997) is consistent with both sets of data and a model that could reconcile the diverse observations would predict that complete blockade of Shh signalling pathway causes

apoptotic cell death, moderate blockade of Shh signalling inhibits proliferation while over activation of Shh pathway results in excessive proliferation.

Consistent with increased Shh signalling being associated with increased proliferate capacity of the cells, analyses of human tumors have revealed mutations in various components of the Shh pathway (reviewed by Wetmore, 2003). Many types of cancer might be caused by inappropriate Shh signalling, the following three were the first associated with Shh signalling: Basal cell carcinoma (BCC) which is a common skin cancer, medulloblastoma (MB) which is a rare but fatal childhood tumor of the cerebellum, and rhabdomyosarcoma (RMS), a muscle tumor. Mutations that inactivate *PTC1* have been identified in all three types of cancer (Goodrich and Scott, 1998), while mutations in *SMO* that make it insensitive to PTC1 repression and mutations in *SU(FU)* that interfere with the export of Gli1 from the nucleus have been identified in BCCs and MBs (Hynes et al., 2000; Reifenberger et al., 1998; Taylor et al., 2002; Xie et al., 1998). Therefore, in addition to the role of Shh as a morphogen required for embryonic patterning of the neural tube and other organs, Shh signalling appears to be clinically important.

1.3 AIMS

Shh signalling functions as a morphogen in the neural tube but how cells perceive intracellularly the differences of the signal strength has not been resolved. Gli proteins, the proposed transcriptional effectors of Shh transduction pathway, have pivotal role in patterning the central nervous system. Moreover, mutations in individual *Gli* genes have limited consequences for ventral neural tube patterning. This raises the possibility that transcription factors other than Gli proteins control the generation of some cell types in the ventral neural tube.

In this study we were interested in understanding the role of Gli proteins controlling cell fate in the ventral neural tube; especially whether and how they mediate graded Shh signalling. More specifically we have attempted to address the following questions:

- Is Gli mediated transcription essential for ventral neural tube patterning?
- Is Gli mediated transcription sufficient to mediate Shh responses in the neural tube?
- Does a gradient of Gli activity recapitulate the patterning effect of graded Shh signalling?
- Is duration of exposure to Shh signalling an important parameter for neural tube patterning?

2. MATERIALS AND METHODS

2.1 GENERAL MOLECULAR BIOLOGY TECHNIQUES

2.1.1 Transformation of chemically competent bacteria

Transformation of the ligated vector or plasmid DNA was performed using chemically competent DH5-α *E. coli* cells that were made according to the protocol available at http://bioprotocol.bio.com/protocolstools/protocol.jhtml?id=p386. Up to 100ng of DNA was added to 100µl of cells thawed on ice. The bacterial cells were kept on ice for 15-30min and then heat shocked at 42°C for 1min followed by cooling in ice for a few minutes. 900µl of LB (Luria-Bertani Broth, 10g/l Tryptone, 5g/l yeast extract, 10g/l NaCl, pH 7.0) was added and the mixture was incubated at 37°C for 1 hour. An aliquot of 100µl from each transformation was spread onto a selective agar plate (0.1mg/ml ampicillin) and incubated overnight at 37°C.

2.1.2 Small scale preparation of DNA

The Plasmid miniprep kit from Biorad or the Qiagen High Speed plasmid midi/maxi kit (Qiagen) was used for all small scale plasmid preparations, according to the manufactures' protocol.

2.1.3 Nucleic acid quantification

Nucleic acid quantification was performed by spectrophotometry at λ = 260 nm, where an optic density (OD) unit corresponds to 50µg/ml of double-stranded DNA or to 40µg/ml single-stranded RNA. The ratio between the readings at λ= 260 nm and λ= 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA should have OD_{260}/OD_{280} ratio =1.8).

2.1.4 Gel electrophoresis

Nucleic acid size separation and size determination were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose (Bio-Rad) in 1x TAE [20mM TRIS acetate, 1mM $Na_2EDTA.2H_2O$ (pH 8.5)] to a final concentration of 1 – 2% (w/v), depending on the expected size of the DNA fragments, and 0.5 mg/ml ethidium bromide. Nucleic acid samples were mixed with 6x gel loading buffer (6x TAE, 50% v/v glycerol, and 0.25% w/v bromophenol blue). Electrophoresis was performed at 5 – 20 V/cm gel length until appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet light ($\lambda \approx 302$nm) and fragment size was estimated by comparison with the 1 kb ladder molecular weight markers (Invitrogen) run in at least one of the gel lanes.

2.1.5 Nucleic acids purification

PCR products were purified with QIAquick PCR Purification kit (Qiagen) and DNA fragments excised from agarose gels were purified with GENECLEAN II KIT (QBIO gene), according to the manufacturers' instructions.

2.1.6 Ethanol precipitation of nucleic acids

Ethanol precipitation of nucleic acids was carried out by adding 1/10 volume of 3M sodium acetate (NaOAc, pH 5.5) and 2.5 volumes of 100% ethanol to the nucleic acid solution. This mixture was left at -20°C overnight. Centrifugation was carried out at 20,000 x g for 30min. The DNA pellet was washed in 70% ethanol and spun again at the same speed for 15 min. After ethanol removal nucleic acid was left to air-dry at room temperature (or at 37°C) for approximately 10 min and re-suspended in distilled H_2O.

2.1.7 DNA Modifications

Restriction digestion, vector de-phosphorylation, filling in and ligation were performed under the recommended conditions by the enzymes supplying companies (Table 1).

Table 1: Enzymes used for DNA modifications.

Reaction	Enzyme and Supplier
Restriction digestion	Wide range of restriction endonucleases by Boehringer Mannheim, Promega, New England Biolabs
De-phosphorylation	Shrimp alkaline phosphatase by Roche
Filling in	DNA Polymerase I Large (Klenow) fragment by Promega
Ligation	T4 DNA Ligase by New England Biolabs

2.1.8 Constructs

All the deletion/ fusion constructs were designed using human *Gli3* (*hGli3*) (Accession number: XP_004833).

Gli3R encodes amino acids 1-768 of hGli3 and it was inserted in the pCAGGS (Niwa et al., 1991) expression vector in the EcoRI restriction site (gift from U. Rüther).

Gli3-ZF-EnR encodes amino acids 471-645 of hGli3 fused to the amino terminus of engrailed repressor domain (Jaynes and O'Farrell, 1991), which is myc tagged at the 3' of EnR sequence. The *hGli3* sequence encoding aa 471-645 was amplified with PCR (High Fidelity PCR Master, Roche) using the following primers:

5' TTT CCA TGG CCA AAC AGG AGC CTG AAG TCA TC 3'
5' TTT GAA TTC GTC CCC TGC CTG CTT CTT G 3'
and program: 2min 94°C
24 cycles 15sec 94°C
30sec 50°C
1min 72°C
followed by 3min 72°C

The product of the PCR (ZF) was subjected to purification, restriction digestion with NcoI and EcoRI, gel electrophoresis, excision of the band from the gel and purification. The DNA fragment was then ligated to linearised (also with NcoI and EcoRI) and purified pSlax vector containing the EnR encoding sequence fused to myc encoding sequence (gift from M. Logan). After a positive clone was identified, plasmid was prepared from it (Slax-Gli3-ZF-EnR), digested with ClaI, Gli3-ZF-EnR fragment was isolated and ligated with linearized (with EcoRI) and dephosphorylated pCAGGS vector after the ends of both DNA fragments were blunted.

RCAS- Gli3-ZF-EnR: (Slax-Gli3-ZF-EnR), digested with ClaI, Gli3-ZF-EnR fragment was isolated and ligated with linearized (also with ClaI) and dephosphorylated RCAS(B) retroviral vector (Fekete and Cepko, 1993).

Gli3-ZF-$VP16_{(4)}$: encodes amino acids 471-645 of hGli3 fused to the amino terminus of four repeats of the minimal VP16 transactivator domain (Ohashi et al., 1994). ZF PCR product ligated, following the same procedure described above for Gli3-ZF-EnR, to linearised (also with NcoI and EcoRI) and purified pSlax vector containing the $VP16_{(4)}$ encoding sequence (gift from M. Logan). After a positive clone was identified, plasmid was prepared from it (Slax-Gli3-ZF-$VP16_{(4)}$), digested with ClaI, Gli3-ZF-$VP16_{(4)}$ fragment was isolated and ligated with linearized (with ClaI) and dephosphorylated bicistronic vector IRES-NLS GFP-pCAGGS, after the ends of both DNA fragments were blunted.

IRES-NLS GFP-pCAGGS: pCAGGS expression vector engineered to bicistronically express nuclear targeted GFP.

LinkerA-IRES-NLS GFP-pCAGGS: pCAGGS expression vector engineered to bicistronically express nuclear targeted GFP, including a sequence containing several restriction sites for easier cloning.

Gli3A$^{\Delta N1}$: encodes amino acids 289-1580 (carboxy terminus) of hGli3 protein tagged at the amino terminus with five repeats of the myc epitope (MEQKLISEEDLNE). The *hGli3* sequence encoding aa 289-745 was amplified with PCR (High Fidelity PCR Master, Roche) using the following primers:

5' TTT CCA TGG CGA GCC GAA AAC GTA CAC TG 3'
5' TTT GAA TTC ATC GAT GGC ACT GAG GTC 3'
and program: 2min 94°C
24 cycles 30sec 94°C
40sec 55°C
4.5min 68°C
followed by 6min 68°C

The PCR product was subjected to purification, restriction digestion with NcoI and EcoRI, gel electrophoresis, excision of the band from the gel and purification. The DNA fragment was then ligated to linearised (also with NcoI and EcoRI) and purified pCS2 vector containing five repeats of the myc sequence 5' of the NcoI site (http://sitemaker.umich.edu/dlturner.vectors/files/cs2_mtpoly.html). After a positive clone was identified, plasmid was prepared from it (pCS2-ΔN1), digested with ClaI, the myc tagged ΔN1 fragment was isolated and ligated with linearized (with ClaI) and dephosphorylated pBluescript (stratagene) containing the sequence encoding aa 744-1580 of hGli3 between the ClaI and XbaI restriction sites. After a positive clone was identified, plasmid was prepared from it (pBl-Gli3A$^{\Delta N1}$), digested with SalI and XbaI, the myc tagged Gli3A$^{\Delta N1}$ fragment was isolated and ligated in the XhoI and NheI restriction sites of LinkerA-IRES-NLS GFP-pCAGGS bicistronic vector.

Gli3A$^{\Delta N2}$: encodes amino acids 468-1580 (carboxy terminus) of hGli3 protein tagged at the amino terminus with five repeats of the myc epitope (MEQKLISEEDLNE). The *hGli3* sequence encoding aa 468-745 was amplified with PCR (High Fidelity PCR Master, Roche) using the following primers:

5' TTT CCA TGG ATG AAA GCA AAC AGG AGC CTG 3'

5' TTT GAA TTC ATC GAT GGC ACT GAG GTC 3'

PCR conditions and following cloning steps were similar to the cloning strategy for Gli3A$^{\Delta N1}$.

pECE-Gli3$^{\Delta N2}$: Gli3$^{\Delta N2}$ was also cloned in the mammalian expression vector pECE (Ellis et al., 1986). pBl-Gli3A$^{\Delta N2}$ was digested with SalI and XbaI, the myc tagged Gli3A$^{\Delta N2}$ fragment was isolated and ligated in the SalI/ XbaI sites of pECE.

GFP was removed from the Gli3$^{\Delta N1}$ and Gli3$^{\Delta N2}$ IRES NLS-GFP pCAGGS in order to use the constructs for protein quantitation assays (these constructs were prepared by Fausto Ulloa).

HoxA7-GFP: sequence encoding nuclear targeted (NLS tagged) GFP (gift from JP Vincent) was cloned at the BamHI restriction site of pBluescript, at the 3' of HoxA7 promoter RI fragment (Gaunt, 2001; Gaunt et al., 1999) which is localised 444 nucleotides 3' to the translation start site of HoxA7. Subsequently, IRES sequence to facilitate bicistronic expression was cloned 3' to the NLS-GFP coding sequence in BamHI restriction site.

HoxA7- Gli3$^{\Delta N1}$: The SV40 polyadenylation sequence (from pCAGGS) was cloned for stability of the transcripts at the 3' of Gli3A$^{\Delta N1}$ in XbaI restriction site of pBl-Gli3A$^{\Delta N1}$ construct. Finally a fragment containing the myc tagged Gli3A$^{\Delta N1}$ sequence and the polyA was cloned at the 3' of the IRES of HoxA7-GFP construct.

HoxA7- Gli3$^{\Delta N2}$: The SV40 polyadenylation sequence (from pCAGGS) was cloned for stability of the transcripts at the 3' of Gli3A$^{\Delta N2}$ in XbaI restriction site of pBl-Gli3A$^{\Delta N2}$ construct. Finally a fragment containing the myc tagged Gli3A$^{\Delta N2}$ sequence and the polyA was cloned at the 3' of the IRES of HoxA7-GFP construct.

HoxA7- Gli3$^{\Delta N1}$ and HoxA7- Gli3$^{\Delta N2}$ were linearized with PvuI, purified with QIAquick Gel Extraction kit (Qiagen) and prepared at 5μg/μl in 10mM Tris.HCl pH 7.4, 0.1mM EDTA for pronuclear injections.

2.1.9 Riboprobe synthesis

For the synthesis of riboprobes, linearised and gel purified plasmid DNA was used. *In vitro* RNA transcription was performed at 37°C for 2 hours using in all cases digoxigenin (DIG)-labeled deoxy-uracil triphosphate (dUTP) (Roche). For a 20μl reaction we used 13μl of linearised plasmid, 2μl of 10x transcription buffer (Roche), 2μl 10x DIG-RNA labelling mix (Roche), 1μl RNase inhibitor (Roche) and 2μl RNA (T3 or T7) polymerase (Roche). Riboprobes were then treated with 20 U DNase I (Roche) at 37°C for 15min to remove DNA template and were purified by size-exclusion chromatography through a Microspin G-50 column (Amersham Biosciences). Using gel electrophoresis (1% agarose gel) the size and integrity of all riboprobes was checked prior to use. Riboprobes were added to hybridisation buffer shortly after synthesis and were stored at -20 °C. Anti-sense RNA probes used are listed in the following table (Table 2).

Table 2. Templates for antisense RNA probes used for *in situ* hybridization

cDNA	**Linearization**	**RNA polymerase**	**Origin**
cPtc1	SalI	T_3	(Pearse et al., 2001)
cPtc2	XbaI	T_3	(Pearse et al., 2001)
cDbx2	NcoI	T_7	(Pierani et al., 1999)
cGsh1	NotI	T_3	ChEST1010I7
cGli1	HindIII	T_3	(Marigo et al., 1996b)

2.2 EMBRYO MANIPULATION AND FUNCTIONAL ASSAYS

2.2.1 Chick *in ovo* electroporation

Plasmid DNA was injected into the lumen of HH st 10-12 (Hamburger, 1951) neural tubes, electrodes placed either side of the neural tube and electroporation carried out using a BTX electroporator delivering five 50 millisecond pulses of 30V (Briscoe et al., 2001). Transfected embryos were allowed to develop and at the indicated time points, embryos were fixed and processed for immunohistochemistry or homogenized in order to produce extracts for the *in vivo* luciferase assay (see below).

2.2.2 Chick and mouse embryo collection

Chick embryo collection: Chick embryos obtained from timed incubation of fertilized eggs were dissected from the eggs, placed in PBS where surrounding membranes were removed.

Mouse embryo collection: Embryos obtained from timed-pregnant surrogate mothers were dissected from the uterus, placed in PBS and subsequently removed from their yolk sac and amnion.

Both chick and mouse embryos collected for staining procedures were transferred to 4% paraformaldehyde (PFA) in PB and left for 1-2 hours at 4°C. They were then washed in PBS, placed in 30% Sucrose in PB and left overnight at 4°C. The next day the embryos were mounted in O.C.T compound (BDH) and subsequently frozen on dry ice. Embryos were stored in -80°C until needed.

2.2.3 Chick *in situ* hybridization on cryosections

In situ hybridisations were performed as described by (Schaeren-Wiemers and Gerfin-Moser, 1993) with a few modifications. Fresh or frozen slides were re-fixed in 4% PFA in PB for 10 minutes and then washed in PBS (3x 3min). The slides were acetylated for

10 minutes [4ml triethanolamine (Fluka) and 0.5ml con. HCl was added to 295ml H_2O and stirred; 0.75ml of acetic anhydride (Sigma) was added just before the sections were immersed] and again transferred to PBS (3x 5min).

Prehybridisation was performed at room temperature with 700µl hybridisation buffer [50% deionised formamide (Sigma), 5x SSC, 5x Denhardts, 10mg/ml herring sperm DNA (Promega), 10mg/ml bakers yeast RNA (Sigma)] per slide from a few hours to overnight in a humidified chamber. The Prehybridisation buffer was replaced with hybridisation mix (200ng of DIG-labelled RNA probe per ml hybridisation buffer). The hybridisation mix was heated for 5min at 80-100°C, to denature the probe, and then chilled on ice before added to the slides. The sections were then covered with coverslips (care was taken to avoid air bubbles) and placed in a humidified chamber (5x SSC/50% formamide) overnight at 70°C.

The next day the slides were washed by placing them vertically in a rack immersed in 5x SCC at room temperature and the coverslips were allowed to slide off. The slides were then washed in 0.2x SCC at 70°C for 1 hour and subsequently adjusted to room temperature in 0.2x SCC for 5min. The slides were transferred to buffer B1 (0.1M Tris.HCl pH 7.5, 0.15M NaCl) for 5min and then blocked for 1 hour at room temperature in buffer B1 with 10% sheep serum. Anti-DIG antibody alkaline phosphatase conjugated (dilution 1:5000) in buffer B1 with 1% sheep serum was added to the slides and left overnight at 4°C in a humidified chamber.

The slides were washed in buffer B1 (3x 5min) and then equilibrated in buffer B3 (0.1M Tris.HCl pH9.5, 0.1M NaCl, 50mM $MgCl_2$). Detection was performed using NBT/BCIP [4µl of 100mg/ml NBT in 70% dimethylformamide (Roche) and 4µl of 50mg/ml BCIP in 70% dimethylformamide (Roche) in 1ml of buffer B3]. 1µl of 2M levamisole was also added to the mixture before it was applied to the slides. The colour reaction was

performed in the dark and stopped by transferring the slides in PBS (minimum 3 long washes). Sections were mounted in Vectashield (Vector) and stored at 4°C.

2.2.4 TUNEL assay on chick cryosections

The assay was performed using the "*In situ* cell death detection kit, fluorescein" (Roche) according to the manufacturer's instructions with the following modification. After rinsing the TUNEL reaction mixture the slides were transferred in B1 buffer (see *in situ* hybridization) blocked for 1 hour at room temperature in buffer B1 with 10% sheep serum. Anti-Fluorescein alkaline phosphatase conjugated antibody (dilution 1:1000) in buffer B1 with 1% sheep serum was added to the slides and left overnight at 4°C in a humidified chamber. The rest of the protocol is identical to the *in situ* hybridization protocol from the antibody incubation onwards.

2.2.5 Immunohistochemistry on chick and mouse cryosections

Blocking buffer (1%BSA / 0.1% Triton in PBS) was added to 12μm thick fresh or frozen sections for 5 minutes. The blocking buffer was then replaced with new containing the primary antibody and left overnight at 4°C. The next day the slides were washed with PBS (4x 5min) and then the secondary antibody was added in blocking buffer. The slides kept in the dark, at room temperature for 1- 2 hours. The slides were washed with PBS (4x 5min). Vectashield (Vector), mounting medium for fluorescence and DAPI, was used for sealing the slides with coverslips. Primary antibodies used are listed in the following table (Table 3). Secondary antibodies FITC, Cy3 and Cy5 conjugated from Jackson Laboratories were used in the appropriate combinations.

Table 3. Primary antibodies used for immunohistochemistry

Epitope/Antigen	Species	Origin
FoxA2	Rabbit	(Ruiz i Altaba et al., 1995b)

Shh	Mouse	(Ericson et al., 1996)
Pax7	Mouse	(Ericson et al., 1996)
Pax6	Mouse	(Ericson et al., 1997b)
Irx3	Rabbit	S. Morton
Nkx6.1	Rabbit	H. Edlund
Nkx2.2	Mouse	(Ericson et al., 1997b)
Olig2	Guinea pig	(Novitch et al., 2001)
Cad7	Mouse	(Nakagawa and Takeichi, 1998)
Msx1	Mouse	(Liem et al., 1995)
Evx1/2	Mouse	S. Morton
En1	Mouse	(Ericson et al., 1997b)
Chox10	Rabbit	(Liu et al., 1994)
Gata3	Mouse	Santa Cruz
MNR2/HB9	Mouse	(Tanabe et al., 1998)
Isl1/2	Mouse	(Ericson et al., 1992)
Cyn1	Mouse	(Ericson et al., 1997b)
Tuj1	Mouse	Convance
HNK-1	Mouse	Becton Dickinson
Laminin	Rabbit	Sigma
GFP	Rabbit	Molecular Probes
GFP	Mouse	Molecular Probes
Myc	Rabbit	(Arber et al., 1999)
Myc	Mouse	Santa Cruz
3C2	Mouse	(Potts et al., 1987)

2.2.6 Image acquisition

High-power images (Normarski) from embryo sections were obtained using a Zeiss Axiophot microscope (Axioplan 2 imaging) fitted with an Axiocam HRC Zeiss digital camera and used Axiovision software. Samples subject to fluorescent immunohistochemistry were imaged on a Leica confocal microscope (True confocal

scanner Leica TCS SP II) with Leica confocal software. Images were processed with Adobe Photoshop.

2.2.7 Quantification of protein levels.

Immunohistochemistry was performed using matched embryo samples as described above using 7 μm thick cryosections. Rabbit anti-myc antibody (Arber et al., 1999) and goat anti-rabbit Alexa Fluor 594 (Molecular Probes) were used to detect the myc tagged $Gli3^{\Delta N1}$ and $Gli3^{\Delta N2}$ proteins. Images were collected on Olympus IX70 inverted microscope with a Prinston Instruments MICROMAX (5 MHz) air cooled interline CCD camera using Softworx image acquisition software (Applied Precision). Intensity of fluorescence was measured in the nuclei of the cells, indicated by DAPI staining, using NIH software Image J. Average intensity of fluorescence per pixel was calculated after normalization for exposure times.

2.2.8 Measurement of Gli transcriptional activity

a. *In vivo* Luciferase assay: SmoM2, Gli3 deletion/fusion constructs or pCAGGS as control were electroporated in chick embryos along with GBS-Luc, a firefly luciferase reporter construct containing eight repeats of the Gli binding sequence (Sasaki et al., 1999) and a Renilla-luciferase reporter carrying the CMV immediate early enhancer promoter (Promega) for normalisation. Embryos were homogenised with a douncer in Passive Lysis Buffer on ice and measurement of Firefly and Renilla luciferase activities was performed using the Dual Luciferase Reporter Assay System (Promega).

b. *In vitro* Luciferase assay: Gli3 deletion/fusion constructs or pCAGGS as control were transfected in C3H/10T1/2 cells (Reznikoff et al., 1973) -using Lipofectamine Plus reagent (Invitrogen)- along with GBS-Luc a firefly luciferase reporter construct

containing eight repeats of the Gli binding sequence (Sasaki et al., 1999) and a Renilla-luciferase reporter carrying the CMV immediate early enhancer promoter (Promega) for normalisation. Preparation of the cell extracts and measurement of Firefly and Renilla luciferase activities was performed using the Dual Luciferase Reporter Assay System (Promega).

Table 4. Formulation of frequently used solutions.

Solution	Formulation
1M PB	0.6M $Na_2HPO_4.7H_2O$, 0.2M $NaH_2PO_4.H_2O$
1x PBS	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM
1x TAE	40mM Tris.Acetate, 2mM $Na_2EDTA.2H_2O$ (pH 8.5)
20x SSC	3M NaCl, 0.3M Na_2citrate.$2H_2O$, adjust pH to 7.0 with 1M HCl
Gel loading Buffer (6x)	6x TAE, 50% v/v glycerol, 0.25% w/v bromophenol blue

3. RESULTS

3.1 Gli activity is required for patterning the entire ventral neural tube.

Analysis of mice lacking Gli2 or Gli3 indicate that Gli proteins are required for a number of aspects of dorsal-ventral patterning, however the generation of MNs and V2 neurons are largely unaffected in both *Gli2* and *Gli3* mutant embryos (Ding et al., 1998; Matise et al., 1998; Park et al., 2000; Persson et al., 2002). This raised the possibility that MN and V2 neuron induction is independent of Gli mediated transcription. To address this issue, we examined neuronal patterning under conditions in which transcription through all Gli proteins was blocked. To accomplish this we designed and tested two inhibitory constructs based on Gli3; these constructs are expected to block all Gli mediated transcription as Gli1, Gli2 and Gli3 recognize common DNA binding sites *in vitro* (Dai et al., 1999; Sasaki et al., 1999).

The first inhibitory construct, Gli3R, encodes a truncated Gli3 protein containing the N-terminal region and zinc finger domain. This is equivalent to the proposed proteolytically processed form of Gli3 (Wang et al., 2000); Fig. 3A). The second inhibitory construct Gli3-ZF-EnR (Fig. 3A), consists of the Gli3 zinc finger domain fused to a widely used transcriptional repressor, the engrailed repressor domain (Jaynes and O'Farrell, 1991). Both Gli3R and Gli3-ZF-EnR were expressed using a vector (pCAGGS) that generates high levels of expression from a chimeric CMV/bActin promoter/enhancer (Niwa et al., 1991).

To test their transcriptional activity we adapted a quantitative assay of Gli activity that uses a Firefly-luciferase reporter gene carrying eight copies of the Gli-binding site from the HNF3β/FoxA2 floor plate enhancer (Sasaki et al., 1997). C3H/10T1/2 cells were co-transfected with the Firefly-luciferase reporter (GBS-Luc), a Renilla-luciferase reporter

carrying the CMV immediate early enhancer promoter (PROMEGA E627A) and either Gli3R, Gli3-ZF-EnR or control pCAGGS plasmid. After 24h the level of Firefly-luciferase was measured and normalized by comparison with level of Renilla Luciferase activity. The level of FF Luciferase activity was five fold lower in the Gli3R and Gli3-ZF-EnR transfected cells compared to the negative control transfected with pCAGGS. These data are consistent with the idea that *in vitro* both Gli3R and Gli3-ZF-EnR block Shh/Gli mediated transcription acting as dominant inhibitory Gli proteins (Fig. 3C).

To test the effect of blocking Gli mediated transcription in the neural tube we ectopically expressed Gli3R at high levels in ventral regions of Hamburger Hamilton stage (HH st) (Hamburger, 1951) 10-12 chick neural tubes using *in ovo* electroporation (Fig. 4,5). A co-electroporated GFP expression plasmid (pEGFP-N1/ Clontech) was used to identify cells transfected with Gli3R.

We first examined whether forced expression of Gli3R affected *Ptc1* and *Ptc2* expression in the neural tube: both genes have been used previously as indicators of Shh signalling (Pearse et al., 2001). Ventrally located cells electroporated with Gli3R exhibited much lower levels of *Ptc1* and *Ptc2* expression than did cells at the same dorsal-ventral position on the control side (Fig. 4Bi-iii). Floor plate formation and Shh production were not affected as indicated by the normal domains of FoxA2 (Fig. 4D) and Shh (Fig. 4C) expression; this was expected since the electroporation method used rarely targets cells of the ventral midline of the neural tube. Normal specification of the floor plate suggested that the reduced level of *Ptc1* and *Ptc2* expression caused by Gli3R results from the loss of response of neural cells to Shh signalling, and not from a decrease in the provision of Shh by the floor plate.

To test directly whether Gli activity is required for D-V patterning throughout the ventral neural tube we examined the expression of progenitor HD proteins and the generation of neuronal subtypes in embryos transfected with Gli3R. In general, ectopic Gli3R had no obvious effect on class I HD proteins in their normal regions of expression; Pax7, *Gsh1*, Irx3 and *Dbx2* were examined and no effect on their expression was identified in the dorsal and intermediate neural tube (Fig. 4E-H). Pax6 expression in the intermediate and dorsal neural tube was also largely unaffected by Gli3R except that in some sections we observed a dorsal shift of the ventral border of Pax6 expression (Fig. 4F). We hypothesize that dowregulation of Ptc1 by Gli3R allows Shh to spread further than normal and thus repress Pax6 at a greater distance from the midline.

However, transfection of Gli3R resulted in the appearance of ectopic Pax7, Pax6, *Gsh1*, Irx3 and *Dbx2* expressing cells ventral to their normal domains of expression (Fig. 4E-H). In the case of Pax7, Pax6 and Irx3 ectopic expressing cells co-expressed GFP indicating that the influence of Gli3R is cell autonomous. Conversely, the expression of the class II proteins Nkx6, Olig2 and Nkx2.2, normally activated in response to Shh signalling, was inhibited in cells transfected with Gli3R (Fig. 5B-D).

Consistent with this ventral-to-dorsal switch in progenitor cell identity, the generation of V0, V1, V2 neurons and MNs was blocked by Gli3R within the normal domains of generation of these neurons (Fig. 5E-H). In addition, the expression of Gli3R resulted in the cell autonomous generation of ectopic V1 neurons ventral to their normal position of generation (Fig. 5F). Thus, Gli3R expression in ventral neural progenitor cells results in cell autonomous changes in the pattern of expression of all progenitor homeodomain proteins examined, corresponding to a ventral-to-dorsal shift in progenitor cell identity. Together, the data indicate that the specification of ventral progenitor cell pattern

requires Gli activity throughout the ventral neural tube and the repression of MN and V2 neuron generation suggests that the generation of these cell types in Gli2 and Gli3 mutant mice is not independent of Gli mediated transcription.

In order to confirm the function of Gli3R as repressor of transcription we assayed the second inhibitory construct, Gli3-ZF-EnR (Fig. 6A). *In vitro*, Gli3-ZF-EnR blocked Shh/Gli mediated transcription acting as a dominant inhibitory Gli protein (Fig. 3C). Unexpectedly, in the neural tube of chick embryos electroporated with Gli3-ZF-EnR very few electroporated cells were detected. Moreover TUNEL analysis indicated a large increase in apoptosis in the electroporated side of the neural tube (Fig. 6H).

We reasoned that this may be a consequence of the high levels of expression generared by the pCAGGS vector. To test this idea we cloned Gli3-ZF-EnR into the chick retroviral vector RCAS(B), which directs lower levels of expression than pCAGGS (James Briscoe, unpublished observation). In the ventral neural tube, electroporation of RCAS-Gli3-ZF-EnR resulted in the appearance of ectopic Pax7, Pax6 and Irx3 expressing cells ventral to the normal domains of expression (Fig. 6B-D). Conversely, the expression of Nkx6, Olig2 and Nkx2.2, normally activated in response to Shh signalling, was inhibited in cells transfected with RCAS-Gli3-ZF-EnR (Fig. 6E-G). The effect of RCAS-Gli3-ZF-EnR was cell-autonomous as only cells expressing Gli3ZnF-EnR (myc immunoreactivity) or retrovirus epitopes (3C2 immunoreactivity) showed changes in gene expression. Thus, Gli3-ZF-EnR blocked Shh signalling in transfected cells in the ventral neural tube resulting in a dorsal to ventral switch in neural progenitor identity. These data support the idea that Gli3R functions as an inhibitor of transcription. Together our data indicate that the specification of ventral progenitor cell pattern requires Gli activity throughout the ventral neural tube.

Figure 3. Transcriptional behaviour of Gli3 repressor candidate constructs.

(A) Diagram summarizing the Gli3R and Gli3-ZF-EnR constructs. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; EnR, Engrailed repressor domain.

(B) Diagram summarizing the reporter construct consisting of eight repeats of Gli binding site (Gli BS) fused to the Luciferase Firefly coding region (Luciferase FF).

(C) Relative luciferase activities of the inhibitory constructs Gli3R and Gli3-ZF-EnR in C3H/10T1/2 cells. Transcription levels in Gli3R and Gli3-ZF-EnR cells are 5 folds lower compared to control. Control corresponds to luciferase activity in pCAGGS transfected cells. I am grateful to Fausto Ulloa for this graph.

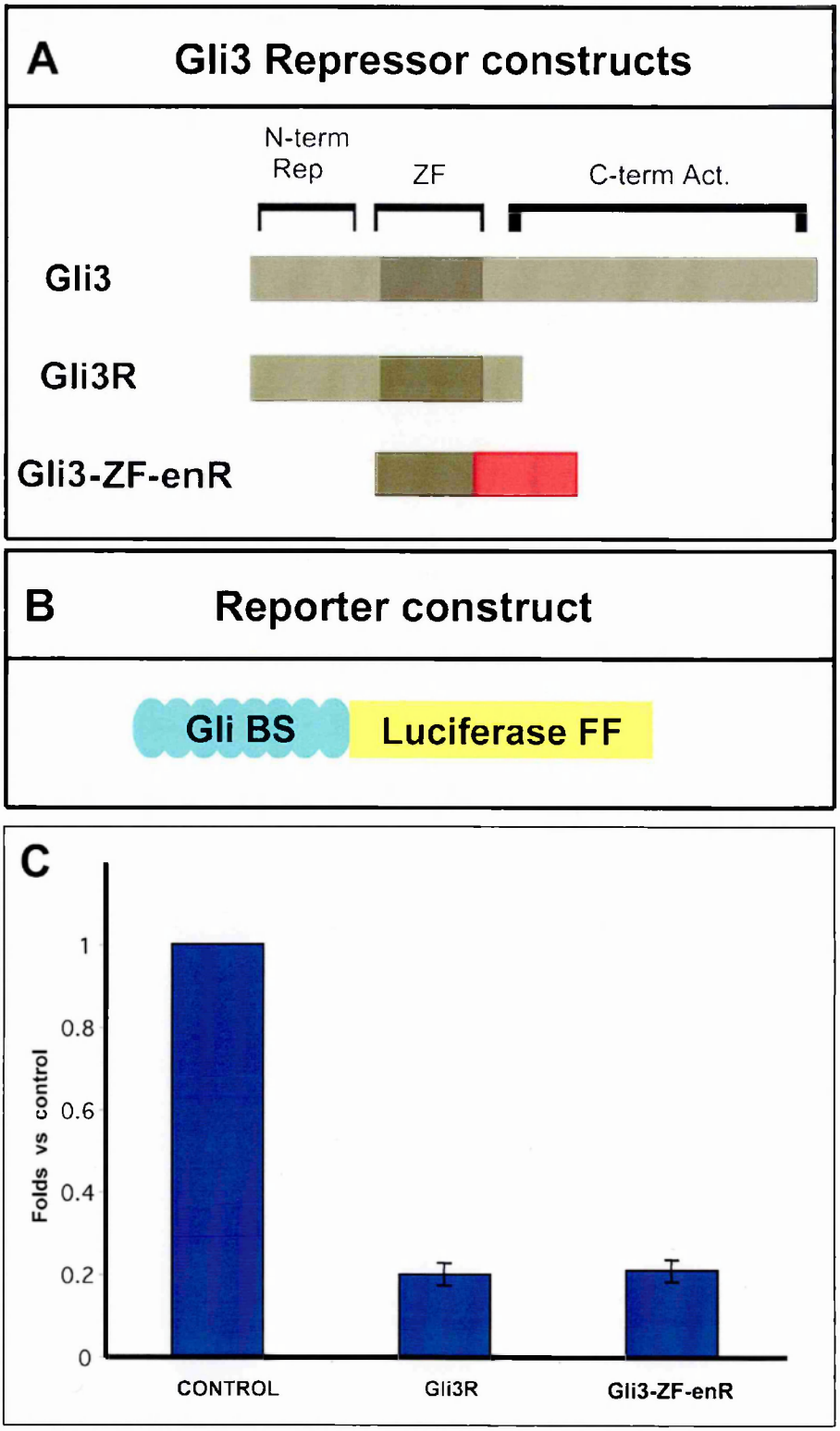

A
Gli3 Repressor constructs
N-term Rep
ZF
C-term Act.
Gli3
Gli3R
Gli3-ZF-enR
B
Reporter construct
Gli BS
Luciferase FF
C
Folds vs control
1
0.8
0.6
0.4
0.2
0
CONTROL
Gli3R
Gli3-ZF-enR

Figure 4. Gli3R dorsalizes the ventral neural tube.

HH stage 10 – 12 embryos were electroporated *in ovo* with Gli3R and GFP, and assayed 24-48h later for the expression of the indicated genes.

(**A**) Diagram summarizing the Gli3R construct. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain.

(**Bi-iii**) Adjacent sections of neural tube showing that expression of *Ptc1* (ii) and *Ptc2* (iii) is reduced in regions of neural tube electroporated with Gli3R as identified by GFP expression (i).

(**C**) Expression of Shh (red) is unaffected in neural tubes transfected with Gli3R (green).

(**D**) Floor plate formation, as indicated by the expression of FoxA2 (red), is unaffected by Gli3R electroporation (green).

(**E-G, Hi-iii**) Ectopic expression of Pax7 (red; E), Pax6 (red; F), Irx3 (red; G), *Dbx2* and *Gsh1* (black arrowheads, Hii and Hiii, respectively) in the ventral neural tube of embryos expressing Gli3R (green). Not all cells that express Gli3R express classI proteins; lateral GFP cells are likely to be neuronal and have down-regulated progenitor markers while the most ventral cells are likely to have been transfected after classI proteins have been inhibited or expressing Gli3R below a threshold necessary to allow classI expression (see Briscoe et al., 2001).

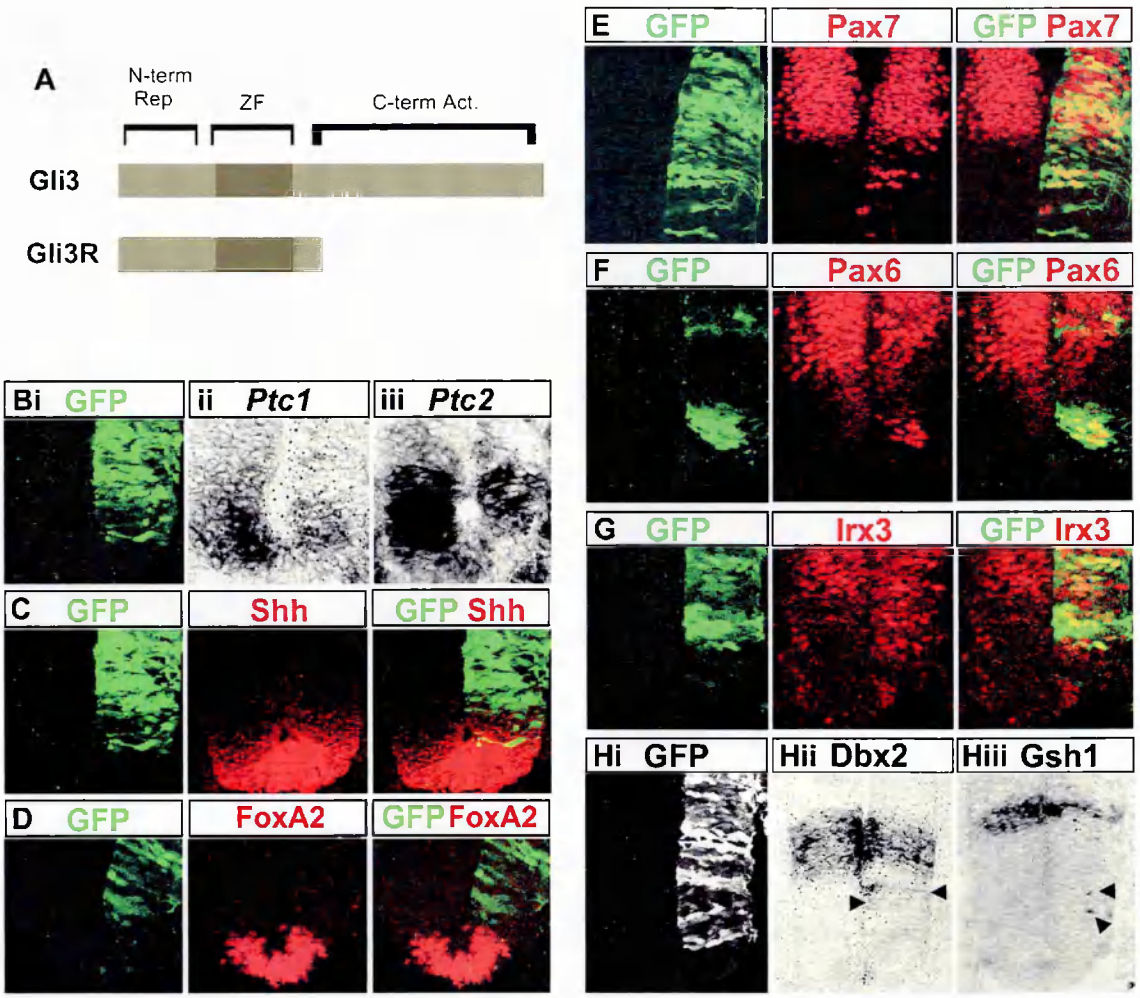

A
N-term Rep
ZF
C-term Act.
Gli3
Gli3R
Bi GFP
ii Ptc1
iii Ptc2
C GFP
Shh
GFP Shh
D GFP
FoxA2
GFP FoxA2
E GFP
Pax7
GFP Pax7
F GFP
Pax6
GFP Pax6
G GFP
Irx3
GFP Irx3
Hi GFP
Hii Dbx2
Hiii Gsh1

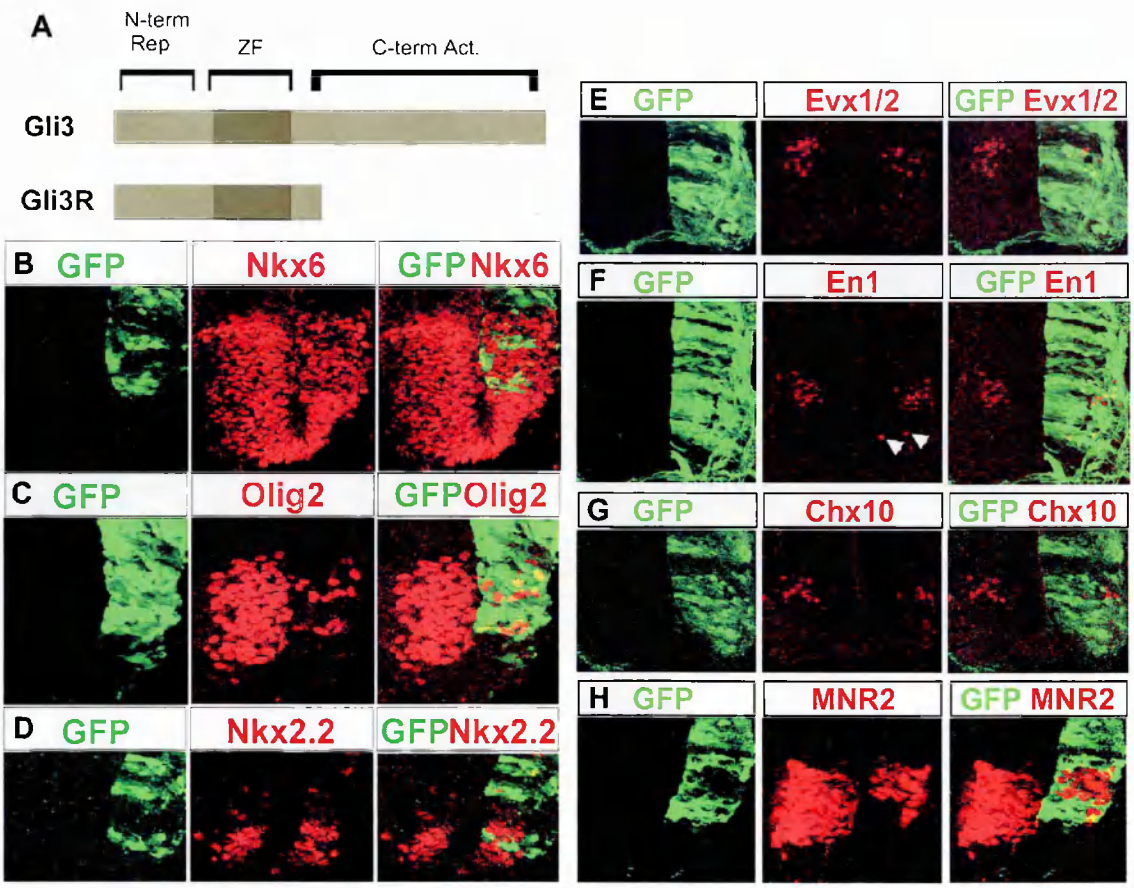
Figure 5. Gli3R blocks the formation of ventral neural tube cell identities.

HH stage 10 – 12 embryos were electroporated *in ovo* with Gli3R and GFP, and assayed 24-48h later for the expression of the indicated genes.

(A) Diagram summarizing the Gli3R construct. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain.

(B-D) Ectopic expression of Gli3R, identified by GFP expression (green) inhibits Nkx6.1/2 (A; red), Olig2 (B; red) and Nkx2.2 (C; red) expression in neural progenitor cells. Some co-expression of Gli3R and class II proteins can be seen, this is likely to represent cells that express Gli3R below a threshold level necessary to inhibit the induction of the class II gene, or cells that have been transfected after the expression of the class II gene has been initiated (see Briscoe et al., 2001).

(E-H) The generation of V0 (E; Evx1/2; red), V1 (F; En1; red), V2 neurons (G; Chx10; red) and MNs (H; MNR2/HB9; red) is inhibited by the expression of Gli3R (green). Occasionally Gli3R electroporation results in the generation of ectopic V1 neurons ventral to their normal position of generation (white arrowheads, F).


A
N-term Rep
ZF
C-term Act.
Gli3
Gli3R
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Nkx6
GFP Nkx6
C GFP
Olig2
GFP Olig2
D GFP
Nkx2.2
GFP Nkx2.2
E GFP
Evx1/2
GFP Evx1/2
F GFP
En1
GFP En1
G GFP
Chx10
GFP Chx10
H GFP
MNR2
GFP MNR2

Figure 6. Gli3-ZF-EnR blocks Shh responses in the ventral neural tube.

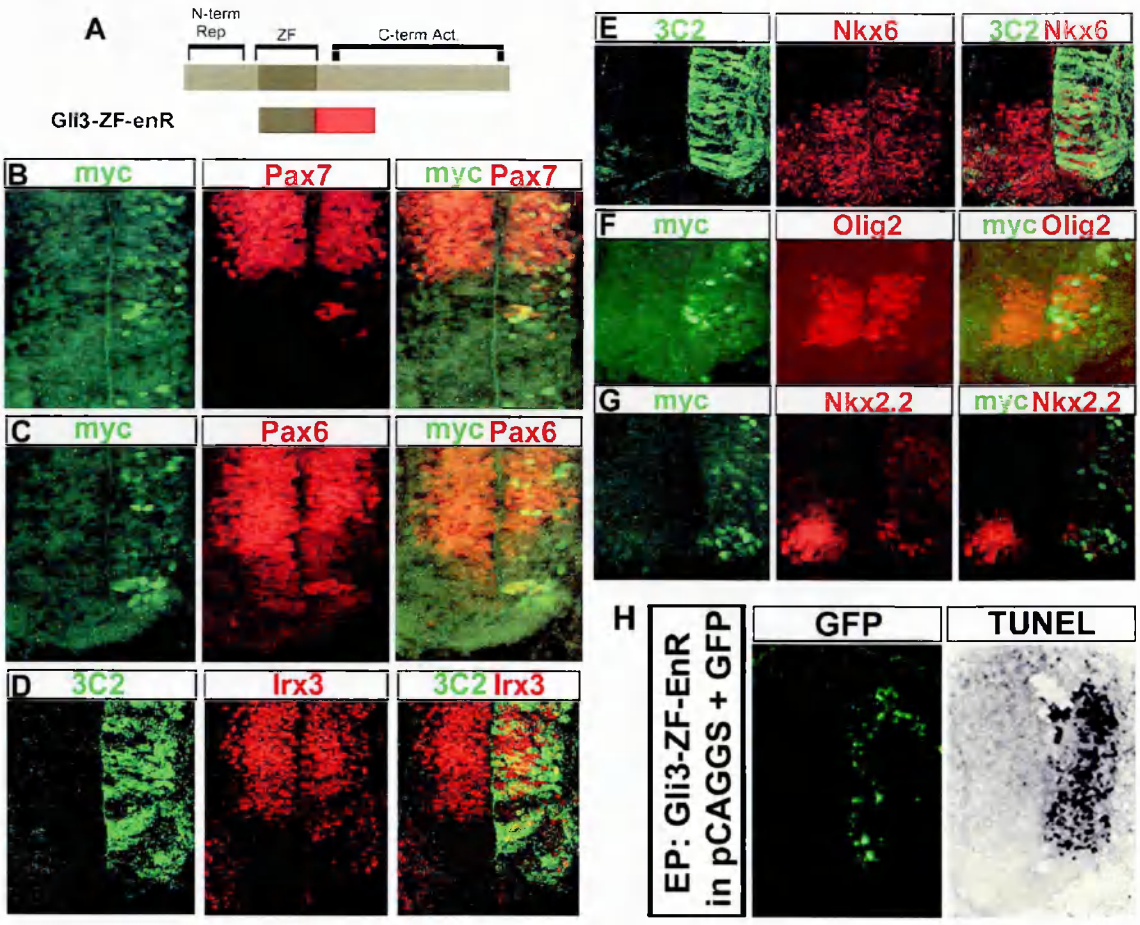
HH stage 10 – 12 embryos were electroporated *in ovo* with RCASGli3ZnF-EnR and assayed 48h later for the expression of the indicated genes.

(A) Diagram summarizing the Gli3-ZF-EnR construct. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; EnR, Engrailed repressor domain.

(B,C,D) Ectopic expression of Pax7 (red; B), Pax6 (red; C) and Irx3 (red; D) in the ventral neural tube of embryos expressing Gli3-ZF-EnR (green). Not all cells that express Gli3-ZF-EnR express Pax7, Pax6, Irx3; lateral green cells are likely to be neuronal and have down-regulated class I proteins while the most ventral cells are likely to have been transfected after class I proteins had been inhibited or expressing Gli3ZnF-EnR below a threshold necessary to allow class I proteins expression (see Briscoe et al., 2001).

(E,F,G) Ectopic expression of Gli3-ZF-EnR (green), identified by staining against myc or retrovirus epitopes (3C2) inhibits Nkx6.1 (E; red), Olig2 (F; red) and Nkx2.2 (G; red) expression in neural progenitor cells. Some co-expression of Gli3-ZF-EnR and class II proteins can be seen, this is likely to represent cells that express Gli3-ZF-EnR below a threshold level necessary to inhibit the induction of the class II gene, or cells that have been transfected after the expression of the class II gene has been initiated (see Briscoe et al., 2001). Apparent co-expression of Gli3-ZF-EnR and Nkx6.1/2 is an artifact caused by high background of Nkx6.1/2 staining; note the background is cytoplasmic, not nuclear.

(H) HH st 10-12 embryos electroporated with pCAGGS Gli3-ZF-EnR and assayed 48h later for apoptosis (TUNEL assay).


A
N-term Rep
ZF
C-term Act.
Gli3-ZF-enR
B myc
Pax7
myc Pax7
C myc
Pax6
myc Pax6
D 3C2
Irx3
3C2 Irx3
E 3C2
Nkx6
3C2 Nkx6
F myc
Olig2
myc Olig2
G myc
Nkx2.2
myc Nkx2.2
H
EP: Gli3-ZF-EnR in pCAGGS + GFP
GFP
TUNEL

3.2 Gli activity is sufficient to induce Shh target genes.

The importance of Gli mediated transcription suggested by the preceding experiments raised the question of whether Gli activity is sufficient to mediate Shh dependent patterning in the neural tube and if a gradient of Gli activity could recapitulate the effect of graded Shh signalling. We focused on Gli3 since it has been shown previously that upon deletion of the amino-terminal repressor domain, Gli3 functions as transcriptional activator (Sasaki et al., 1999).

We prepared and assayed three constructs based on human Gli3 which we predicted to activate Shh target genes independent of Shh signalling, Gli3-ZF-VP16$_{(4)}$, Gli3A$^{\Delta N1}$ and Gli3A$^{\Delta N2}$. Gli3-ZF-VP16$_{(4)}$ comprises of the DNA binding domain (zinc fingers) of Gli3 fused to four repeats of the VP16 transactivation domain (Ohashi et al., 1994). In Gli3A$^{\Delta N1}$ and Gli3A$^{\Delta N2}$ the first 288aa or the entire region amino-terminal of the DNA binding domain (aa1-470) of Gli3 were replaced with five repeats of Myc epitope (Myc5, see Materials and Methods; Fig. 7A). All three constructs were expressed using pCAGGS vector, which was engineered to express bicistonically the Gli3 activator (Gli3A) and nuclear targeted GFP, a marker used to identify transfected cells. Dominant Gli activators are expected to activate transcription *in vitro*.

We tested the Gli transcriptional activity of the "activator" constructs using *in vitro* luciferase assay (see Materials and Methods). C3H/10T1/2 cells were co-transfected with the Firefly-luciferase reporter (GBS-Luc), a Renilla-luciferase reporter for normalization and either Gli3A$^{\Delta N1}$, Gli3A$^{\Delta N2}$, Gli3-ZF-VP16$_{(4)}$ or control pCAGGS plasmid. 24h after transfection the level of Firefly-luciferase was measured and normalized by comparison with level of Renilla Luciferase activity. All three constructs functioned as

transcriptional activators as the levels of luciferase activity increased between 17-33 folds compared to the control, pCAGGS transfected cells (Fig. 7B).

In order to assess the effect of the activator constructs in the neural tube patterning these were electroporated *in ovo*. Here I focus on the analysis of Gli3A$^{\Delta N1}$ and Gli3A$^{\Delta N2}$; Gli3-ZF-VP16$_{(4)}$ effect on patterning is described in section "3.6 Alternative approaches to analyze graded Gli activity". To examine whether Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ alters the response of neural cells to Shh signalling we tested whether the expression of either protein changes the level of expression of chick *Ptc1, Ptc2* and *Gli1* in the neural tube, which have previously been used as indicators of Shh signalling (Pearse et al., 2001). 24h after electroporation of HH st10-12 chick embryos with Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$, the untransfected side of the neural tube expressed chick *Ptc1, Ptc2* and *Gli1* at high levels ventrally and at lower levels dorsally (Fig. 8B-G; (Goodrich et al., 1996; Marigo et al., 1996b). On the electroporated side, dorsal cells that expressed either Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ exhibited a much higher level of chick *Ptc1, Ptc2* and *Gli1* expression than did cells at the same dorsoventral position on the control side (Fig. 8B-G). The increase in endogenous chick *Ptc1, Ptc2* and *Gli1* expression appeared to be confined to groups of cells expressing Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ (Figs. 8B-G). This is similar to the effect of transfecting an activated Smoothened construct, SmoM2 (Fig. 8H-J; (Hynes et al., 2000). These data are consistent with previous results (Sasaki et al., 1999) and suggest that Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ act as dominant active Gli proteins and are sufficient to mediate Shh responses.

Figure 7. Transcriptional behaviour of Gli3 activator candidate constructs.

(A) Diagram of the deletion/fusion Gli constructs. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; VP16, VP16 transactivation domain.

(B) Relative luciferase activities of Gli3-ZF-$VP16_{(4)}$, $Gli3^{\Delta N1}$ and $Gli3^{\Delta N2}$ in C3H/10T1/2 cells. All three constructs function as activators of transcription generating many folds higher transcriptional activity compared to the control. Control corresponds to luciferase activity in pCAGGS transfected cells. I am grateful to Fausto Ulloa for this graph.

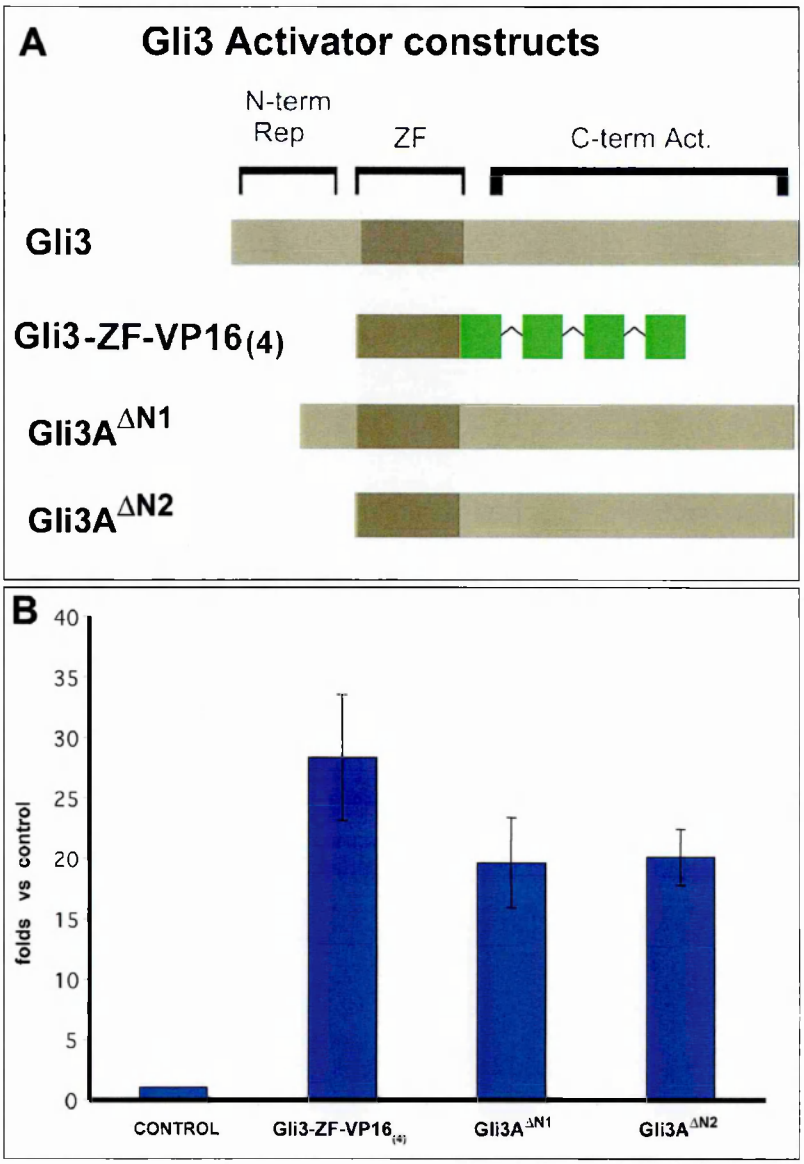
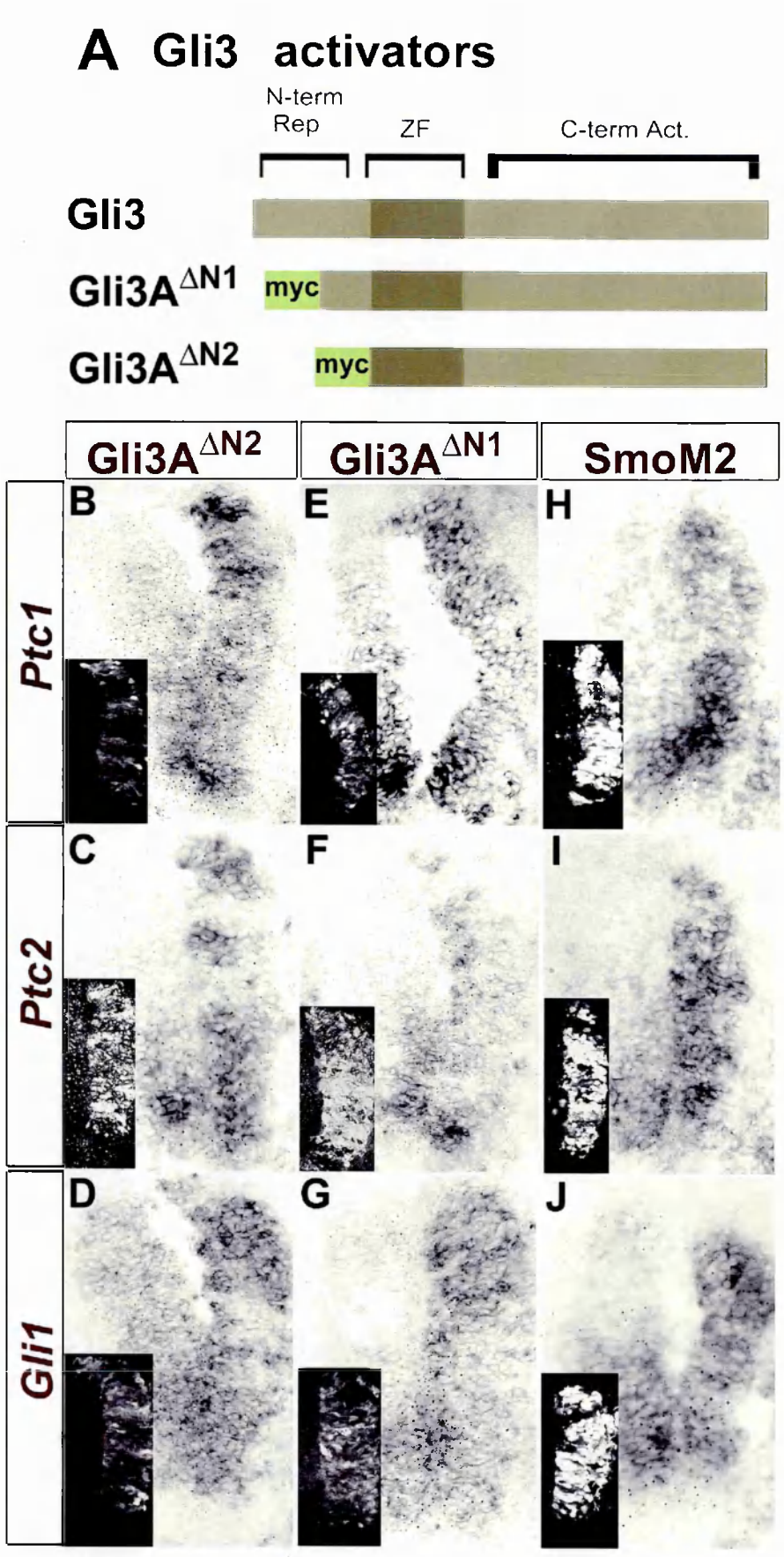

A
Gli3 Activator constructs
N-term Rep
ZF
C-term Act.
Gli3
Gli3-ZF-VP16(4)
Gli3A^ΔN1
Gli3A^ΔN2
B
folds vs control
40
35
30
25
20
15
10
5
0
CONTROL
Gli3-ZF-VP16(4)
Gli3A^ΔN1
Gli3A^ΔN2

Figure 8. Gli activators upregulate expression of Shh target genes.

(**A**) Gli activator constructs were generated by deleting part ($Gli3^{\Delta N1}$) or all ($Gli3^{\Delta N2}$) of the amino terminal/repressor domain of human Gli3 (hGli3). Diagram summarizing hGli3, $Gli3^{\Delta N1}$ and $Gli3^{\Delta N2}$. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; Myc, 5 repeats of Myc epitope.

(B-J) Electroporation with $Gli3^{\Delta N2}$, $Gli3^{\Delta N1}$ and SmoM2, a constitutively active form of Smoothened, results in the upregulation of Hh signalling targets *Ptc1, Ptc2* and *Gli1* on the transfected side of the neural tube. HH st 10-12 embryos were electroporated with $Gli3^{\Delta N2}$ (B-D), $Gli3^{\Delta N1}$ (E-G), or SmoM2 (H-J) and assayed 24h later for the expression of *Ptc1* (B,E,H), *Ptc2* (C,F,I), *Gli1* (D,G,J). All sections are from the forelimb and anterior thoracic region of transfected embryos. Electroporated side is on the right and electroporation efficiency is indicated either by Myc immunofluorescence (insets B-G) or by GFP immunofluorescence (insets H-J).


A Gli3 activators
N-term Rep
ZF
C-term Act.
Gli3
Gli3A^ΔN1
myc
Gli3A^ΔN2
myc
Gli3A^ΔN2
Gli3A^ΔN1
SmoM2
Ptc1
Ptc2
Gli1
B
C
D
E
F
G
H
I
J

3.3 Different levels of Gli activity induce different Shh responses

3.3.1 Activated Gli constructs generate different levels of Gli activity *in vivo*.

We noticed that cells transfected with Gli3A$^{\Delta N2}$ appeared to consistently express higher levels of *Ptc1, Ptc2* and *Gli1* than those transfected with Gli3A$^{\Delta N1}$. This raised the possibility that Gli3A$^{\Delta N2}$ functions as a stronger activator than Gli3A$^{\Delta N1}$. To test this we adapted the luciferase reporter assay. HH st 10-12 chick embryos were co-electroporated with the GBS-Luc, a normalization plasmid (Renilla Luciferase reporter) and either Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ (see Materials and Methods; Fig. 9B). After 24h incubation the level of Firefly-luciferase was measured in neural cells and normalized by comparison with level of Renilla Luciferase activity. Both Gli3A$^{\Delta N1}$ and Gli3A$^{\Delta N2}$ activated the GBS-Luc reporter (Fig. 9C) however Gli3A$^{\Delta N1}$ consistently induced two to three fold lower levels of activity than Gli3A$^{\Delta N2}$ (n=8). These data are consistent with the idea that both Gli3A$^{\Delta N1}$ and Gli3A$^{\Delta N2}$ act as dominant active constructs and indicate that Gli3A$^{\Delta N1}$ has two to three fold lower activity than Gli3A$^{\Delta N2}$.

To extend the range of activity generated by the activated Gli3 constructs we sought to generate lower levels of Gli activity by using a different expression vector. We found that the mammalian expression vector pECE (Ellis et al., 1986) which contains an early SV40 promoter directs significantly lower levels of expression in chick neural tube cells than pCAGGS (M.Cheung, pers comm; Fig. 9A). Taking advantage of the Myc5 epitope present in Gli3A$^{\Delta N2}$ we compared protein levels in the nuclei of pCAGGS-Gli3A$^{\Delta N2}$ and pECE- Gli3A$^{\Delta N2}$ electroporated cells. Neural cells transfected with pECE-Gli3A$^{\Delta N2}$ contained 4-6 fold lower levels of Gli3A$^{\Delta N2}$ protein than cells tranfected with pCAGGS-Gli3A$^{\Delta N2}$ (Fig. 9A). In addition pECE-Gli3A$^{\Delta N2}$ electroporated cells generated 5-7 fold lower levels of GBS-Luc reporter activity than cells transfected with pCAGGS-Gli3A$^{\Delta N2}$ and 2-3 fold lower activity than Gli3A$^{\Delta N1}$ transfectants (Fig. 9C, n=3). Together these

constructs pCAGGS-Gli3A$^{\Delta N2}$, pCAGGS-Gli3A$^{\Delta N1}$ and pECE- Gli3A$^{\Delta N2}$ provide the means to generate a range of Gli transcriptional activity that differ by increments of 2-3 fold and we refer to these constructs as Gli3A^{HIGH}, Gli3A^{MED}, Gli3A^{LOW}, respectively (Fig. 9D).

3.3.2 High levels of Gli activity induce the most ventral cell types in the neural tube.

To examine whether Gli activity is sufficient to mediate dorsal-ventral patterning of the ventral neural tube we assayed the expression of progenitor domain and neuronal subtype markers in embryos transfected with each Gli3A construct. To allow informative comparisons between constructs and transfected embryos we have standardized the conditions used in these experiments. To this end, HH st 11-12 embryos were electroporated with each construct and incubated for 48h, analysis was restricted to the forelimb and anterior thoracic regions and embryos that were poorly transfected or lacked ectopic expression in dorsal or intermediate regions of the neural tube were excluded from the analysis. For clarity we have divided the spinal cord into four territories: Region A, encompasses the floor plate and progenitors (p) of V3 neurons; Region B comprises the progenitors pMN and p2 that generate MNs and V2 neurons situated dorsal to p3 and ventral to Dbx2 expression; Region C includes the progenitor domains p1, p0, dI6 that express Dbx2; and Region D contains the progenitor domains dI5-dI1 dorsal to the region of Dbx2 expression (Fig. 2; (Persson et al., 2002).

First we focused on Region A. Floor plate is marked by FoxA2 expression while p3 progenitors and their progeny V3 neurons express Nkx2.2 (Fig. 2). Forced expression of Gli3A^{HIGH} resulted in the ectopic expression of FoxA2 and Nkx2.2 throughout the dorsal-ventral axis (Fig. 10B, C) and the repression of Pax6 (Fig. 11B). Cells expressing markers of Region A in ectopic positions co-expressed GFP and were positive for the

Myc5 epitope present in Gli3A^{HIGH} indicating the effects of Gli3A^{HIGH} were cell autonomous. Not all progenitor cells that expressed Gli3A^{HIGH} coexpressed either Nkx2.2 or FoxA2 (Fig. 10B, C). We noticed that the higher the level of Myc5 immunoreactivity in the transfected cell, the greater was the probability of ectopic Nkx2.2 or FoxA2 expression, thus ectopic induction of Region A markers appears to depend on high levels of Gli3A^{HIGH} (see below).

In contrast to Gli3A^{HIGH} ectopic expression of Gli3A^{MED} was not sufficient to induce FoxA2 or Nkx2.2 in the intermediate neural tube (Fig. 10E, F). However, cells transfected with Gli3A^{MED} contained decreased levels of Pax6 (Fig. 11H) and the expression of Gli3A^{LOW} and Gli3A^{MED} in the most dorsal region of the neural tube adjacent to the roof plate occasionally resulted in a small number of ectopic FoxA2 expressing cells (Fig. 10F,G) - a region of the neural tube that has been previously characterized as more competent than other regions of the neural tube for floor plate induction (Ruiz i Altaba et al., 1995a). These data are consistent with the idea that Gli3A^{MED} has activator function but it does not have sufficient activity to induce ectopic FoxA2 or Nkx2.2 in the intermediate neural tube. Together these data indicate that a high level of Gli activity is sufficient to reprogramme intermediate neural tube cells to express markers characteristic of the most ventral region of the neural tube.

We noticed that in many cases both Gli3A^{HIGH} and Gli3A^{MED} caused elongation of the electroporated side of the neural tube (Fig. 10A-C). Hh signalling has been associated with increased proliferation capacity of the cells (Duman-Scheel et al., 2002; Kenney et al., 2003). We reasoned that activating Shh signalling by Gli3A^{HIGH} and Gli3A^{MED} increased the proliferation of the electroporated cells, resulting in a longer neural tube (Fig. 10E; see below).

3.3.3 Moderate levels of Gli activity induce motor neurons

We next turned our attention to Region B which expresses Olig2, HB9/MNR2 and Nkx6.1 but not Pax7. This region encompasses the domain that generates MNs and V2 neurons (Fig. 2). Consistent with the ability of $Gli3A^{HIGH}$ to induce ventral neural tube identities, mosaic expression of $Gli3A^{HIGH}$ in cells situated dorsal to Region B resulted in the cell autonomous ectopic expression of Olig2, HB9/MNR2 and Nkx6.1 and repression of Pax7 and Irx3 (Fig. 10A and Fig. 11A, D, F). It was noticeable however, that, in general, the transfected cells that expressed Region B markers expressed lower levels of $Gli3A^{HIGH}$ than the cells expressing Region A markers (Fig 10; see below). Within Region B itself, expression of high levels of $Gli3A^{HIGH}$ appeared to cause the cell autonomous extinction of expression of both Olig2 and HB9/MNR2 (Fig. 10A and Fig. 11F). This correlated with the ectopic expression of Nkx2.2 and FoxA2 in this region (Fig. 10B, C) indicating that these cells had acquired a more ventral identity.

In contrast to the inability of $Gli3A^{MED}$ to induce Region A markers, Region B fates were induced by ectopic expression of $Gli3A^{MED}$ in the intermediate neural tube. Mosaic expression of $Gli3A^{MED}$ resulted in the cell autonomous induction of Olig2 (Fig. 10D) and Nkx6.1 (Fig. 11J) and the repression of the dorsal marker Pax7 (Fig. 11G). In addition, many $Gli3A^{MED}$ transfected cells contained low levels of Pax6 and had repressed expression of Irx3 (Fig. 11H, I). Consistent with the progenitor code induced by $Gli3A^{MED}$, transfected embryos contained ectopic MNs and V2 neurons identified by HB9/MNR2 and Chx10 expression, respectively (Fig. 11K, L). These data suggest that compared to $Gli3A^{HIGH}$ the 2-3 fold lower levels of Gli activity provided by $Gli3A^{MED}$ are sufficient to induce MNs and V2 neurons but not Region A fates.

3.3.4 Low levels of Gli activity induce intermediate neural tube fates.

To address whether lower levels of Gli activity had an effect on neural tube patterning we analyzed embryos transfected with $Gli3A^{LOW}$. First we tested the effect of $Gli3A^{LOW}$ on the three most ventral cell identities: floor plate ($FoxA2^{+}$), p3 ($Nkx2.2^{+}$) and pMN ($Olig2^{+}$). With the exception that FoxA2 was induced in the roof plate in a small number of embryos, $Gli3A^{LOW}$ electroporated cells did not upregulate any of these markers (Fig. 12I-L). These data suggest that $Gli3A^{LOW}$ has activator function but of not sufficient levels to induce ectopic FoxA2 or Nkx2.2 in the intermediate neural tube.

$Gli3A^{LOW}$ did not induce Olig2, but ectopic expression of Nkx6.1 (Fig. 12H) in the intermediate neural tube was occasionally observed in transfected embryos. The expression of Nkx6.1 in the absence of pMN markers is indicative of p2 identity, consistent with this ectopic V2 neurons were occasionally observed (Fig. 12G). These data suggest that a lower level of Gli activity is required for V2 neuron production than for MN induction.

We next examined markers of the intermediate neural tube, specifically the region that contains the progenitors of V1, V0 and dI6 neurons. This is ventral to Msx expression and encompasses progenitors that express Dbx2 and Cad7, the dorsal half of this region expresses Pax7 (Fig. 2). We reasoned that $Gli3A^{LOW}$ might provide sufficient Gli activity to induce characteristics of Region C. Consistent with this forced expression of $Gli3A^{LOW}$ induced expression of *Dbx2* and Cad7 in Region D (Fig. 12C,D). Concomitantly $Gli3A^{LOW}$ repressed Msx1 (Fig. 12A) while Pax7 expression was repressed in some but not all $Gli3A^{LOW}$ expressing cells (Fig. 12B). Progenitors expressing *Dbx2* in the absence of Pax7 generate V1 and V0 neurons, consistent with this, ectopic generation of V1 and V0 neurons in more dorsal positions was observed in

Gli3A^{LOW} transfected embryos (Fig. 12E,F). Together these data indicate that low levels of Gli activity are sufficient to induce characteristics of the intermediate neural tube in more dorsal regions of the neural tube. Overall, the data support the idea that a gradient of Gli activity simulates graded Shh signalling.

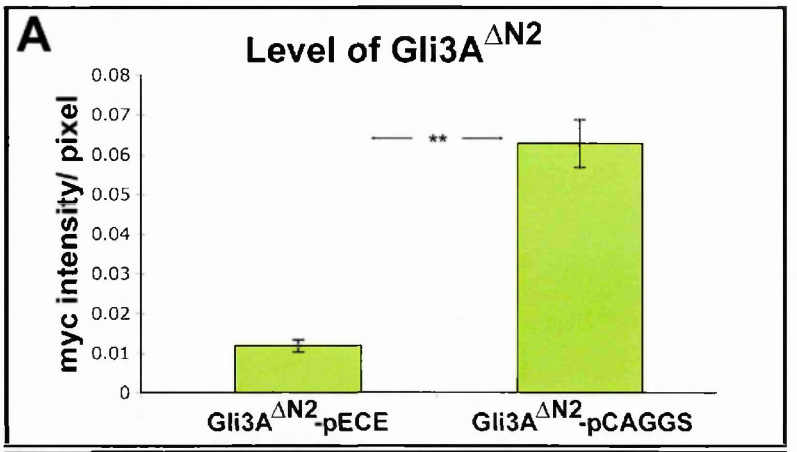
Figure 9. Activated Gli constructs generate different levels of Gli transcriptional activity *in vivo*.

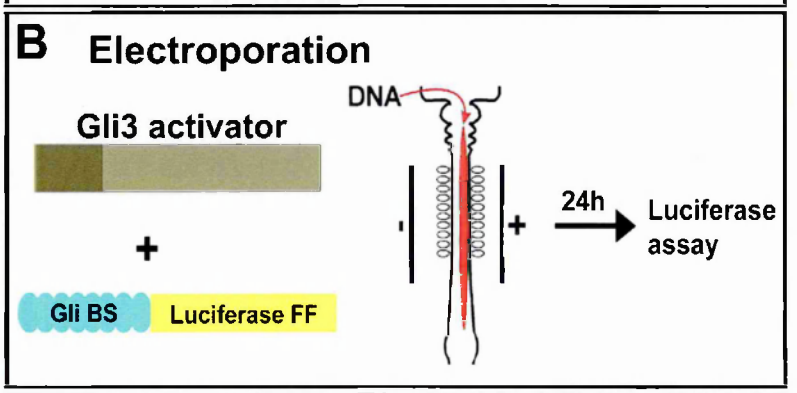
(A) HH st 11-12 embryos were electroporated with the indicated constructs. $Gli3^{\Delta N2}$ expressed using two different vectors pECE and pCAGGS. Quantification of protein levels in chick neural cells transfected with pECE-$Gli3^{\Delta N2}$ had ~5 fold lower levels of $Gli3^{\Delta N2}$ than cells in embryos electroporated with pCAGGS-$Gli3^{\Delta N2}$. Graph (A) shows the relative Myc immuno-reactivity (average intensity of fluorescence per pixel), indicating the levels of Myc tagged $Gli3^{\Delta N2}$ protein ± S.E.M in the nuclei of neural tube cells of chick embryos electroporated with pCAGGS- $Gli3^{\Delta N2}$ and pECE-$Gli3^{\Delta N2}$. pECE-$Gli3^{\Delta N2}$ σ= 0.007; pCAGGS-$Gli3^{\Delta N2}$ σ= 0.037; **Student's t-test P= 1.63×10^{-10}.

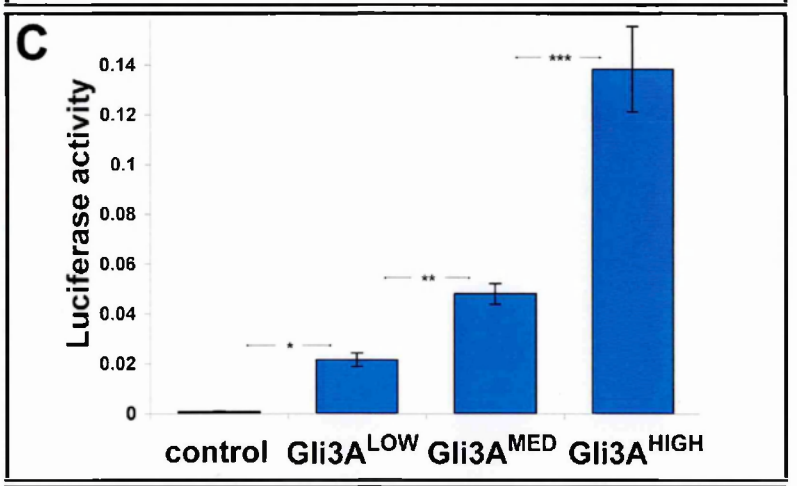
(B) HH st 10-12 embryos were electroporated with each activator together with the reporter construct containing eight repeats of the Gli binding site (Sasaki et al., 1997) and normalisation plasmid, and assayed 24h later for Luciferase activity.

(C) $Gli3A^{LOW}$, $Gli3A^{MED}$ and $Gli3A^{HIGH}$ generate low, moderate and high levels of transcriptional activity *in vivo*. Graph shows the Luciferase activity of $Gli3A^{LOW}$, $Gli3A^{MED}$ and $Gli3A^{HIGH}$ ± S.E.M. $Gli3A^{LOW}$ σ= 0.008; $Gli3A^{MED}$ σ= 0.012; $Gli3A^{HIGH}$ σ= 0.049. Student's t-test: *control-$Gli3A^{LOW}$ P= 4.715×10^{-5}; **$Gli3A^{LOW}$-$Gli3A^{MED}$ P= 0.0002; ***$Gli3A^{MED}$-$Gli3A^{HIGH}$ P= 0.001.

(D) pCAGGS-$Gli3A^{\Delta N2}$, pCAGGS-$Gli3A^{\Delta N1}$ and pECE-$Gli3A^{\Delta N2}$ were named according to their efficacy in the *in vivo* luciferase assay $Gli3A^{HIGH}$, $Gli3A^{MED}$ and $Gli3A^{LOW}$ respectively.







D Table of Gli3 activators

Gli3A^{HIGH}	= Gli3A$^{\Delta N2}$-pCAGGS
Gli3A^{MED}	= Gli3A$^{\Delta N1}$-pCAGGS
Gli3A^{LOW}	= Gli3A$^{\Delta N2}$-pECE

Figure 10. High levels of Gli transcriptional activity induce Region A markers

Different levels of Gli transcriptional activity induce different sets of ventral neural markers. $Gli3A^{HIGH}$ electroporation induced ectopic expression of the two most ventral identities, p3 progenitors and floor plate cells (FP). $Gli3A^{MED}$ electroporation induced MN progenitors but not p3 or FP.

HH st 11 – 12 embryos were electroporated *in ovo* with $Gli3A^{HIGH}$ (A-C) and $Gli3A^{MED}$ (D-G) and assayed 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of embryos.

(a-g) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-F) marking expression of Olig2 (A,D), Nkx2.2 (B,E), FoxA2 (C,F,G).

(A-C) Olig2 (A), Nkx2.2 (B), FoxA2 (C) were ectopically expressed in dorsal and intermediate regions of neural tube electroporated with $Gli3A^{HIGH}$.

(D-F) Olig2 (D), but not Nkx2.2 (E) or FoxA2 (F) were induced in $Gli3A^{MED}$ transfected cells.

(G) In a small number of $Gli3A^{MED}$ electroporated embryos we occasionally observed a small number of ectopic $FoxA2^{+}$ cells, mainly close to the roof plate.

Note that both $Gli3A^{HIGH}$ and $Gli3A^{MED}$ produced a noticeable increase in proliferation on the transfected side of electroporated neural tubes.

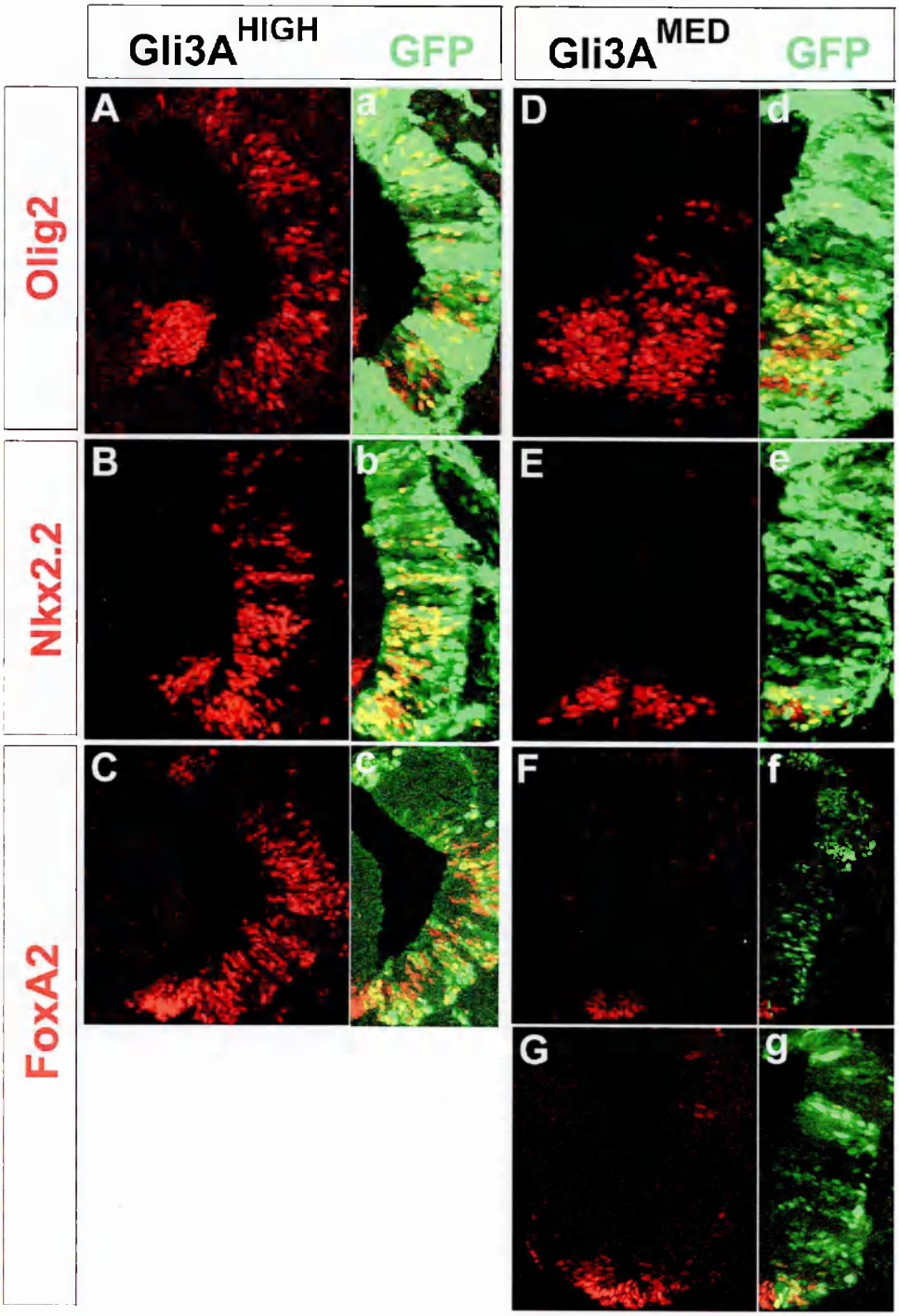

Gli3A^HIGH
GFP
Gli3A^MED
GFP
Olig2
Nkx2.2
FoxA2
A
a
D
d
B
b
E
e
C
c
F
f
G
g

Figure 11. High and moderate levels of Gli transcriptional activity induce MNs and V2 interneurons.

In addition to Region A markers, Gli3A^{HIGH} induces MNs and V2 neurons. Gli3A^{MED} was also sufficient to induce MNs and V2 neurons.

HH st 11–12 embryos were electroporated *in ovo* with Gli3A^{HIGH} (A-F) or Gli3A^{MED} (G-L) and assayed 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of the embryos.

(a-l) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-L) marking expression of the indicated markers.

(A, G) Pax7 is downregulated in both Gli3A^{HIGH} and Gli3A^{MED} electroporated cells. There are a small number of Pax7 expressing cells ventral to the normal boundary of expression, this may be a consequence of upregulation of *Ptc* (Figure 8) causing a non autonomous induction of Pax7 (Briscoe et al., 2001).

(B, C, H, I) Pax6 (B, H) and Irx3 (C,I) are downregulated in the majority of Gli3A^{HIGH} (B,C) transfected cells, but are downregulated only in a proportion of Gli3A^{MED} (H,I) transfected cells.

(D-F,J-L) Nkx6 (D,J), Chx10 (E,K) and MNR2/HB9 (F,L) are ectopically expressed by Gli3A^{HIGH} (D-F) and Gli3A^{MED} (J-L) electroporated cells.

Note that a monoclonal anti-GFP antibody is used in e, k which does not readily detect low levels of GFP expression.

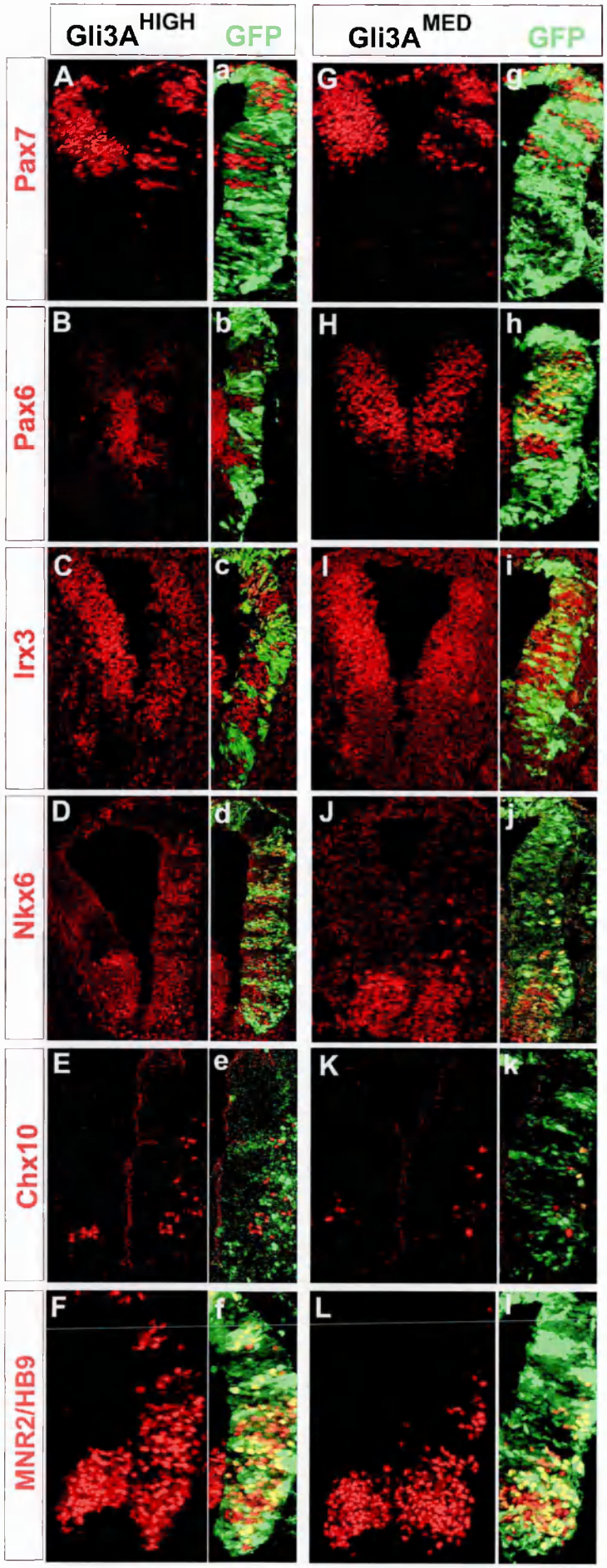

Gli3A HIGH
GFP
Gli3A MED
GFP
Pax7
Pax6
Irx3
Nkx6
Chx10
MNR2/HB9
A
a
G
g
B
b
H
h
C
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Figure 12. Low levels of Gli activity induce cell fates characteristic of the intermediate neural tube.

HH st 11–12 embryos were electroporated *in ovo* with $Gli3A^{LOW}$ (A-K) and assayed 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of the embryos.

(a, b, d-k) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A, B, D-K) marking expression of the indicated markers.

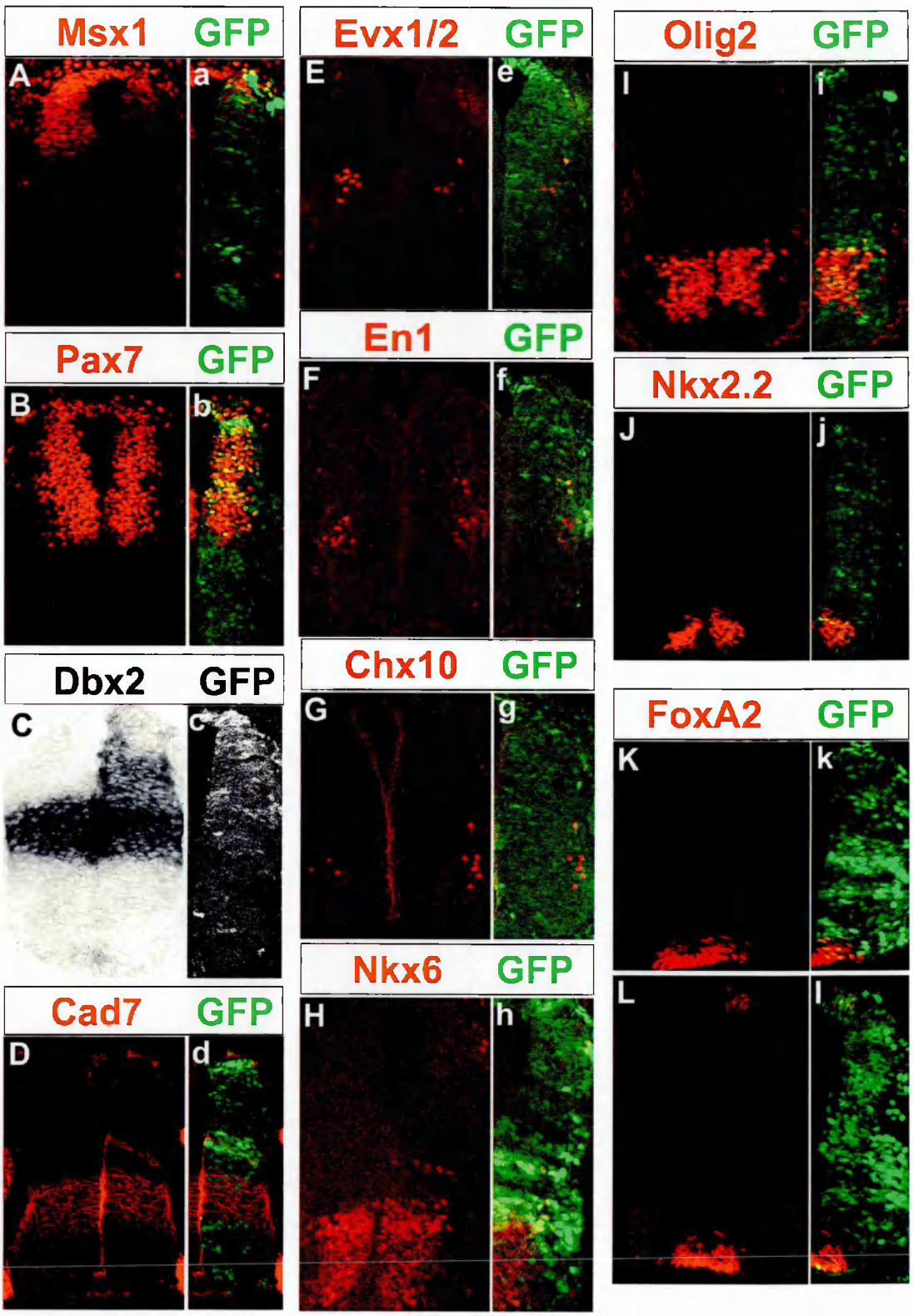
(c) GFP immunofluorescence indicating the electroporated cells of the *Dbx2* hybridized section

(A) Msx1 is downregulated in $Gli3A^{LOW}$ expressing cells.

(B) Pax7 is largely unaffected. In a small number of $Gli3A^{LOW}$ electroporated cells Pax7 is downregulated.

(C-H) $Gli3A^{LOW}$ electroporation causes the ectopic expression of *Dbx2* (C), Cad7 (D), Evx1/2 (E), En1 (F), Chx10 (G) and Nkx6 (H) in regions dorsal to their normal boundaries of expression.

(I-L) Olig2 (I), Nkx2.2 (J), FoxA2 (K) were restricted to their normal regions of expression in $Gli3A^{LOW}$ electroporated neural tubes. $Gli3A^{LOW}$ did not induce the three most ventral cell types (pMN, p3, FP). Occasionally FoxA2 is ectopically expressed by $Gli3A^{LOW}$ electroporated cells at or next to the roof plate, but not in cells of the intermediate neural tube (L).


Msx1 GFP
A
a
Pax7 GFP
B
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Dbx2 GFP
C
c
Cad7 GFP
D
d
Evx1/2 GFP
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F
f
Chx10 GFP
G
g
Nkx6 GFP
H
h
Olig2 GFP
I
i
Nkx2.2 GFP
J
j
FoxA2 GFP
K
k
L
l

3.4 Activator Gli proteins function independent of Shh signalling and are sufficient to maintain the fidelity of a graded Shh response.

3.4.1 Gli activators induce Shh responses in the absence of ongoing Shh signalling.

We next tested the idea that activated Gli proteins are sufficient to mediate Shh responses in the absence of ongoing Shh signalling. We first considered the possibility that the responses induced by the Gli3A constructs reflected an increase in the production of Shh itself. In particular, the induction of FoxA2 by $Gli3A^{HIGH}$ raised the possibility that Shh induction was responsible for the activities attributed to this construct. To address this issue we examined Shh expression. *In ovo* electroporation of $Gli3A^{HIGH}$, $Gli3A^{MED}$ and $Gli3A^{LOW}$ did not alter Shh expression at 24hpt (hours after electroporation) (Fig. 13A-C). At 48hpt, neither $Gli3A^{MED}$ nor $Gli3A^{LOW}$ induced Shh expression in ventral or intermediate regions of the neural tube (Fig. 13D, E). In contrast expression of $Gli3A^{HIGH}$ resulted in a small number of cells ectopically expressing Shh, this is consistent with the observed induction of FoxA2 (Fig. 10C).

To rule out the possibility that ectopic Shh expression mediated the induction of ventral markers, we analyzed embryos that had been co-electroporated with Gli3A constructs and the extracellular inhibitor of Shh signalling HIP, which is a transmembrane glycoprotein that binds all three vertebrate Hedgehog proteins and blocks Hedgehog signalling (Chuang and McMahon, 1999). Expression of HIP on its own resulted in the repression of pMN and p3 markers Olig2 and Nkx2.2 respectively and the ventral expansion of Pax7, marker of dorsal identities (Fig. 14A,B,C). As indicated by FoxA2 expression, floor plate formation was largely unaffected (Fig. 14D). Co-electroporation of $Gli3A^{HIGH}$ and HIP did not affect the activity of $Gli3A^{HIGH}$. In co-transfected embryos, $Gli3A^{HIGH}$ expression resulted in the cell autonomous induction of FoxA2, Nkx2.2 and Olig2 (Fig. 14F-H) and the repression of Pax7 while adjacent untransfected

cells displayed the non-autonomous HIP induced ventral-to-dorsal shift in identity, evidenced by ectopic Pax7 expression (Fig. 14E). Similarly, the activity of $Gli3A^{MED}$ was unaffected by the co-electroporation with HIP. Co-transfected cells expressed Olig2 and repressed Pax7 (Fig. 15A,B). Moreover, ectopic V1 and V0 neurons were induced in embryos transfected with $Gli3A^{LOW}$ and HIP (Fig. 15F,G). These data provide evidence that the effects of the activated Gli3 constructs result from an increase in the response of neural cells to Shh signalling, and not from an increased provision of Shh by the floor plate or ectopic induction of Shh expression. Moreover the activity of these dominant active constructs is cell autonomous and does not require ongoing Shh signalling in neural cells. Together these findings indicate that Gli activity is sufficient to mediate the effects of Shh signalling in responding cells.

3.4.2 The level of Gli activity correlates with changes in neuronal patterning.

Transfection of each Gli3A construct resulted in the induction of genes corresponding to more than one of the defined progenitor domains. $Gli3A^{HIGH}$ induced FP, p3, pMN; $Gli3A^{MED}$ induced pMN and p2 and $Gli3A^{LOW}$ induced p1, p0 and dI6. One possibility that could account for these data is that ectopic expression of the Gli3A constructs results in the specification of cell types with mixed identities such that individual cells transfected with a Gli3A construct express combinations of genes not normally encountered *in vivo*. To test this possibility we examined whether the fidelity of progenitor domain and neuronal subtype identity was maintained in cells transfected with Gli3A constructs.

We first focused on $Gli3A^{HIGH}$ transfected embryos and assayed the expression pMN and p3 markers at 48hpt. Embryos transfected with $Gli3A^{HIGH}$ showed ectopic expression of

Olig2 and Nkx2.2 and repression of Pax7, individual transfected cells did not coexpress Olig2 with Pax7 (Fig. 16A). Moreover Olig2 was not co-expressed with Nkx2.2 at these times points (Fig. 16B). These data indicate that MN and Region A identity remains distinct and intact in these experiments. Next we examined Nkx2.2 and FoxA2 expression. Approximately 20-30% of Gli3A^{HIGH} transfected cells that expressed Nkx2.2 also expressed FoxA2 (Fig. 16C). This is consistent with expression of Nkx2.2 in floor plate cells at early developmental stages (Ericson et al., 1997a) and the ability of Nkx2.2 to induce FP and FoxA2 expression (M. Persson and J. Ericson pers. com.). These data raise the possibility that FP induction by Gli3A^{HIGH} is indirect and proceeds via Nkx2.2 induction.

We next turned our attention to Gli3^{MED} and Gli3A^{LOW}. Ectopic induction of Olig2 by Gli3A^{MED} correlated with the repression of Pax7 and co-expression of Olig2 with Pax7 was not observed (Fig. 16D). Moreover, Isl1 and Chx10 were not co-expressed in Gli3A^{MED} transfected embryos (Fig. 16E). Finally we examined Gli3A^{LOW} transfected embryos and consistent with the preceding data we did not observe co-expression of V1 and V2 markers En1 and Chox10, respectively (Fig. 16F). Taken together, these data provide evidence that progenitor and neuronal subtype gene expression codes remain intact in these experiments. This suggests that activation of Gli mediated transcription is sufficient to faithfully recapitulate Shh signalling.

An alternative explanation for the multiple progenitor domain markers and neuronal subtypes generated by each Gli3A construct is that this is a consequence of the different levels of transfected protein in individual cells, resulting from the stochastic nature of *in ovo* electroporation. If this were the case then the level of Gli3A protein expressed in a

cell should correspond with the genes being expressed in that cell. To test this possibility, we took advantage of the Myc5 tag present in the $Gli3^{\Delta N2}$ to quantify the level of protein in individual transfected cells and correlate this with marker gene expression. We first compared embryos transfected with $Gli3A^{HIGH}$ with embryos expressing $Gli3A^{LOW}$. Cells transfected with $Gli3A^{LOW}$ contained ~6 fold lower levels of $Gli3A^{\Delta N2}$ than cells in embryos electroporated with $Gli3A^{HIGH}$ (Fig. 9A; Fig. 16G). We extended this analysis in embryos transfected with $Gli3A^{HIGH}$. We divided transfected cells into a group that expressed Nkx2.2 and a group that expressed Olig2. We found that the levels of $Gli3A^{\Delta N2}$ were approximately 50% higher in cells expressing ectopic Nkx2.2 than cells expressing Olig2 (Fig. 16G). These data support the idea that the multiple progenitor identities induced by each of the activated Gli proteins reflects the level of protein expression in individual cells. Moreover the data suggest that a gradient of Gli activity is sufficient to mediate graded Shh signalling and indicate that small differences in the level of activated Gli is sufficient to control differential gene expression. Thus, the 2-3 fold changes in Shh concentration that are necessary to switch between alternative neuronal subtypes appear to be mediated by relatively small changes in the level of Gli activation.

Figure 13. Gli3A^{HIGH} but not Gli3A^{MED} or Gli3A^{LOW} is sufficient to induce ectopic Shh expression.

HH st 11–12 embryos were electroporated *in ovo* with Gli3A^{HIGH} (A, D), Gli3A^{MED} (B, E) and Gli3A^{LOW} (C, F) and assayed 24h (A-C) or 48h (D-F) later for the expression of Shh. All sections are from the forelimb and anterior thoracic region of the embryos.

(a -f) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A, B, D-K) marking expression of the indicated markers.

(A-C) At 24hpt none of the Gli3A constructs are sufficient to induce ectopic Shh.

(D) At 48hpt ectopic Shh was detected in a small number of sections from Gli3A^{HIGH} electroporated embryos.

(E, F) Gli3A^{MED} and Gli3A^{LOW} did not induce ectopic expression of Shh 48hpt.

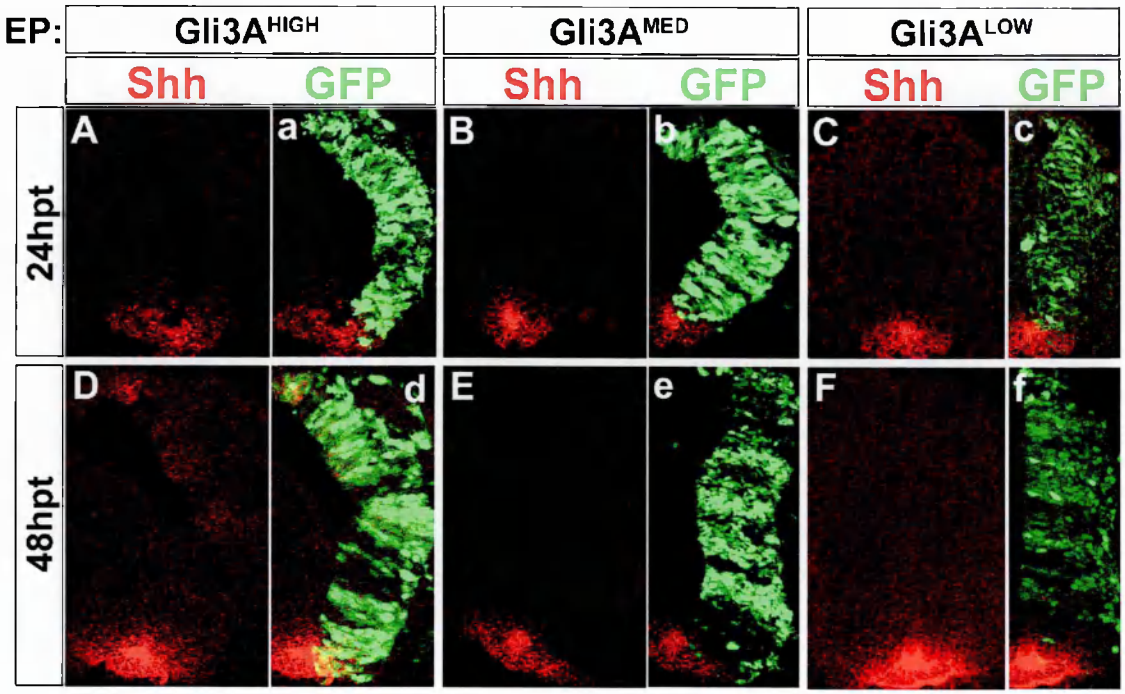

EP:
Gli3A^HIGH
Gli3A^MED
Gli3A^LOW
Shh
GFP
Shh
GFP
Shh
GFP
24hpt
48hpt
A
a
B
b
C
c
D
d
E
e
F
f

Figure 14. High Gli3A activator functions independent of Shh signalling.

$Gli3A^{HIGH}$ activator can function in the absence of ongoing Hh signalling. Co-electroporation of $Gli3A^{HIGH}$ with HIP does not block the induction of ventral markers by $Gli3A^{HIGH}$.

HH st11–12 embryos were co-electroporated *in ovo* with GFP and HIP (A-D) or $Gli3A^{HIGH}$ and HIP (E-H) and assayed 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of the embryos.

(a-h) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-H) showing expression of the indicated markers.

(A) Overexpression of HIP results in the ventral expansion of Pax7.

(B, C) Olig2 (B) and Nkx2.2 (C) expression was downregulated by HIP electroporation in a non cell autonomous manner.

(D) Expression of FoxA2 was not affected, suggesting that formation of floor plate is not affected by HIP electroporation.

(E) Co-electroporation of $Gli3A^{HIGH}$ and HIP results in the cell autonomous repression of Pax7 in the electroporated cells. Pax7 is ectopically expressed in non electroporated cells in the ventral neural tube.

(F-H) Olig2 (F), Nkx2.2 (G) and FoxA2 (H) are ectopically expressed in the intermediate and dorsal neural tube by $Gli3A^{HIGH}$ and HIP co-electroporated cells.

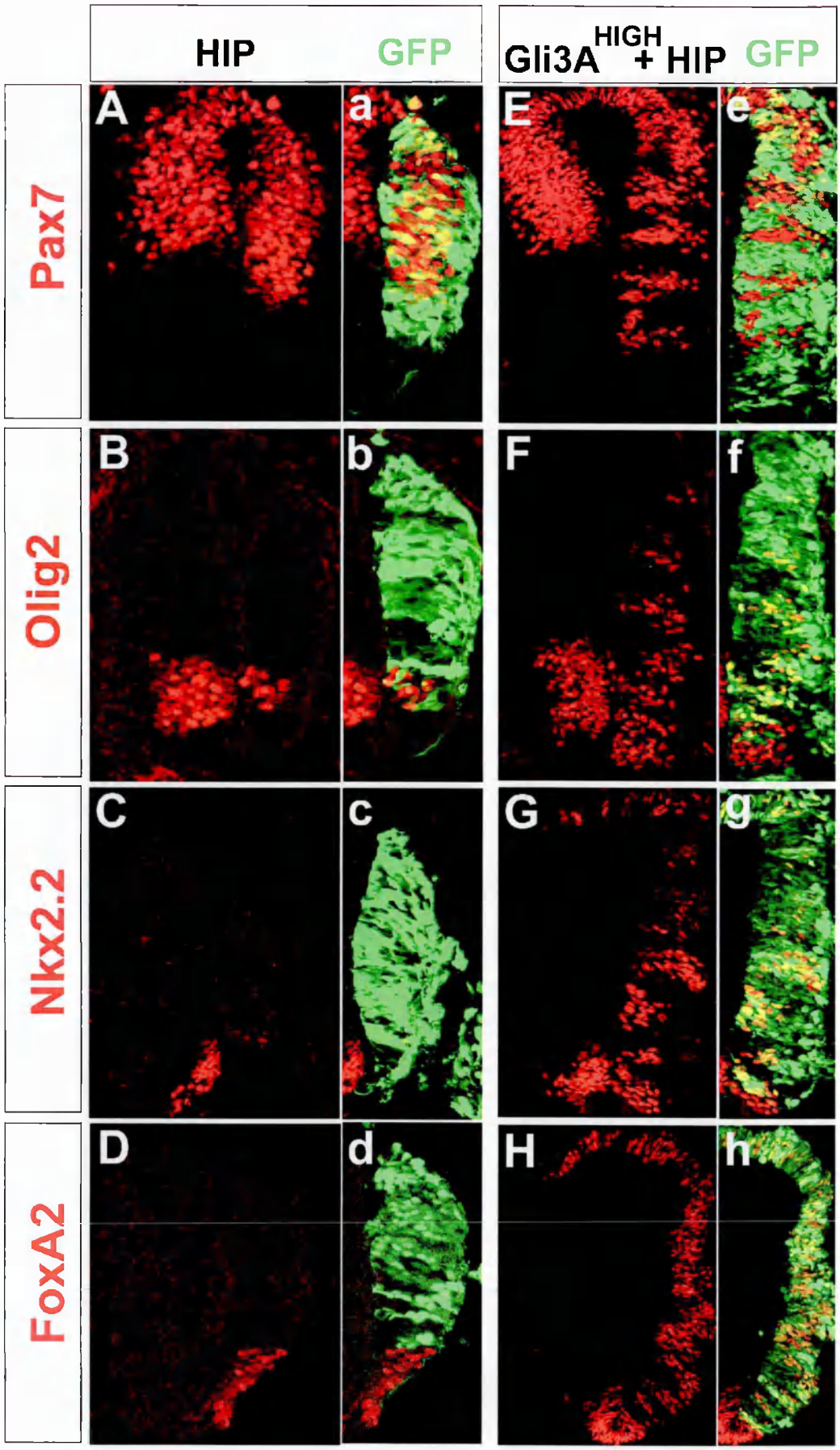

HIP
GFP
Gli3A HIGH + HIP
GFP
Pax7
Olig2
Nkx2.2
FoxA2
A
a
E
e
B
b
F
f
C
c
G
g
D
d
H
h

Figure 15. Moderate and low Gli3A activators function independent of Shh signalling.

$Gli3A^{MED}$ and $Gli3A^{LOW}$ activators can function in the absence of ongoing Hh signalling. Co-electroporation of $Gli3A^{MED}$ or $Gli3A^{LOW}$ with HIP does not block the induction of ventral and intermediate markers by $Gli3A^{MED}$ and $Gli3A^{LOW}$, respectively.

HH st11–12 embryos were co-electroporated *in ovo* with $Gli3A^{MED}$ and HIP (A-D) or $Gli3A^{LOW}$ and HIP (E-H) and assayed 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of the embryos.

(a-t) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-T) showing expression of the indicated markers.

(A, E) Co-electroporation of $Gli3A^{MED}$ and HIP (A) results in the cell autonomous repression of Pax7 in the electroporated cells. Pax7 is ectopically expressed in non electroporated cells in the ventral neural tube. Co-electroporation of $Gli3A^{LOW}$ and HIP (E) results in the cell autonomous repression of Pax7 in a small number of electroporated cells. Note, Pax7 is ectopically expressed in the ventral neural tube.

(B-D) Olig2 (B) is ectopically expressed dorsal to its normal boundary by $Gli3A^{MED}$ and HIP co-transfected cells. As is the case with $Gli3A^{MED}$ electroporations Nkx2.2 (C) and FoxA2 (D) were restricted to their normal regions of expression.

(F, G) Ectopic V0 neurons (F; Evx1/2) and V1 neurons (G; En1) were generated dorsal to their normal region by $Gli3A^{LOW}$ and HIP co-transfected cells.

(H) Consistent with the $Gli3A^{LOW}$ electroporation, Nkx2.2 expression was restricted ventrally in the $Gli3A^{LOW}$ and HIP co-electroporated embryos.

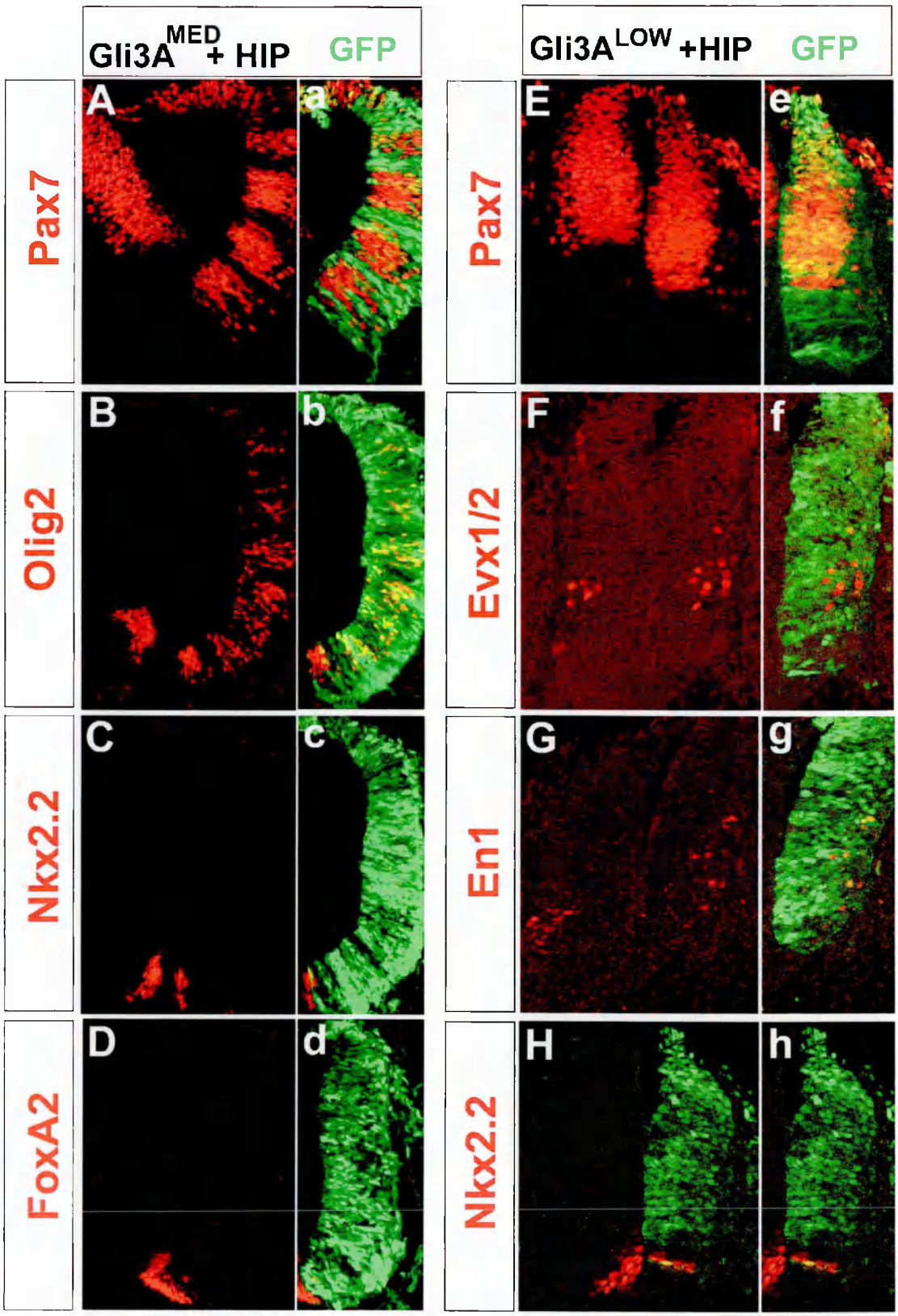

Gli3A MED + HIP
GFP
Gli3A LOW +HIP
GFP
Pax7
Olig2
Nkx2.2
FoxA2
Pax7
Evx1/2
En1
Nkx2.2
A
a
B
b
C
c
D
d
E
e
F
f
G
g
H
h

Figure 16. The level of Gli activity correlates with changes in neuronal patterning

Gli activity is sufficient to faithfully recapitulate Shh signalling, as transfected cells do not co-express markers characteristic of different progenitor domains or neuronal subtypes. In addition, ectopic Nkx2.2 cells have higher amount of Gli activator in the nucleus compared to the ectopic Olig2 cells.

HH st 11–12 embryos were electroporated *in ovo* with Gli3A^{HIGH} (A-C, G), Gli3A^{MED} (D, E) or Gli3A^{LOW} (F) and assayed 24h or 48h later for the expression of the indicated genes. All sections are from the forelimb and anterior thoracic region of the embryos.

(Ai-iii) Pax7 is downregulated in Gli3A^{HIGH} electroporated cells and Pax7 is not co-expressed with ectopically induced Olig2.

(Bi-iii) Olig2 and Nkx2.2 are ectopically expressed in different Gli3A^{HIGH} transfected cells.

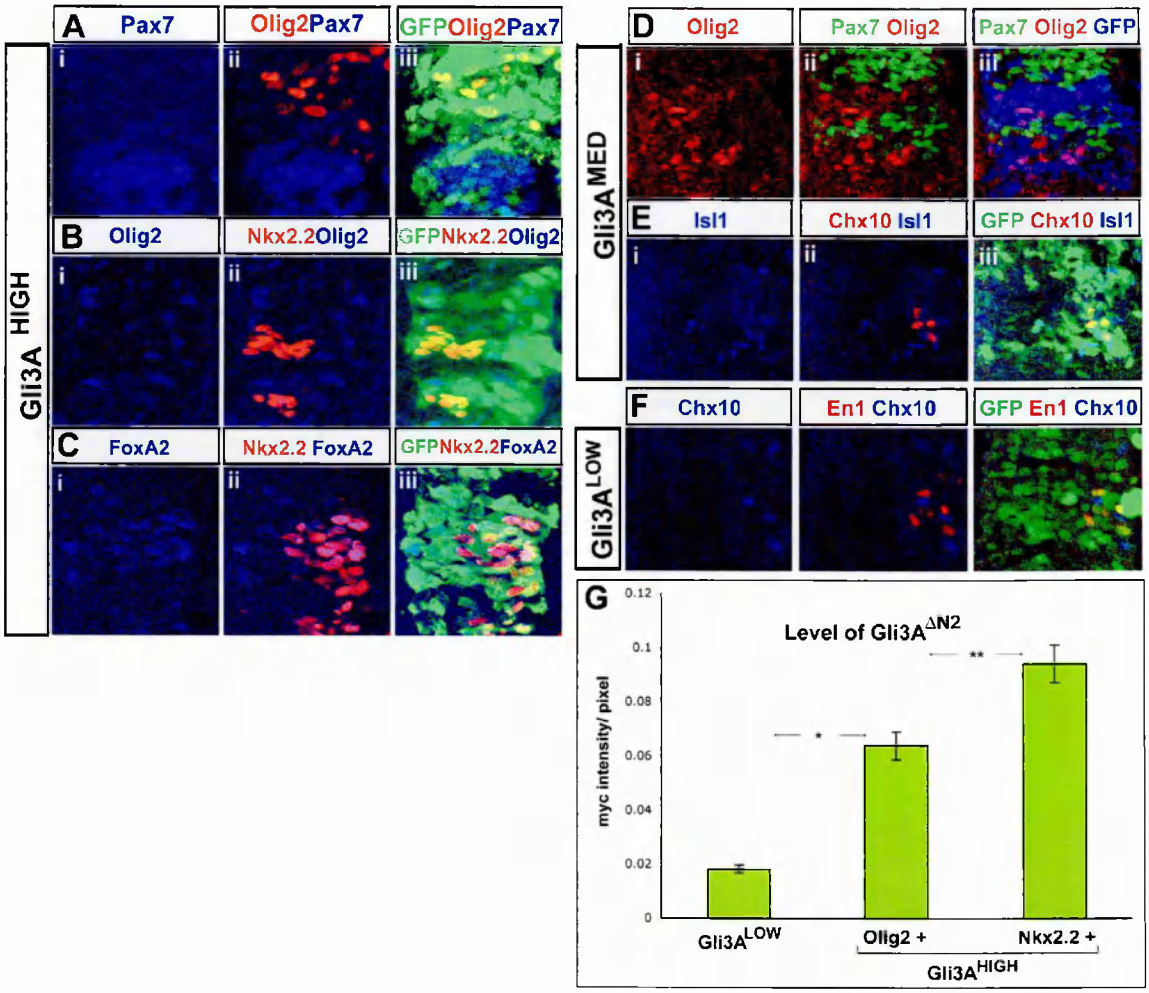
(Ci-iii) Nkx2.2 and FoxA2 expression is upregulated in Gli3A^{HIGH} transfected cells and they co-localize in approximately 25% of the ectopic Nkx2.2 positive cells.

(Di-iii) Pax7 is downregulated by Gli3A^{MED} electroporated cells and is not co-expressed with ectopically induced Olig2.

(Ei-iii) Gli3A^{MED} transfected cells form ectopic MNs (Isl1) or V2 neurons (Chx10) and do not co-express markers of both neuronal subtypes.

(F) Gli3A^{LOW} transfected cells form ectopic V1 neurons (En1) or V2 neurons (Chx10) and do not co-express markers of both neuronal subtypes.

(G) Quantification of Gli3A$^{\Delta N2}$ protein levels in chick neural cells transfected with Gli3A^{LOW} or Gli3A^{HIGH}. Graph shows the relative Myc immuno-reactivity (average intensity of fluorescence per pixel) ± S.E.M, indicating the levels of Myc tagged Gli3$^{\Delta N2}$ protein in the nuclei of cells. For Gli3A^{HIGH} cells were divided into those expressing Nkx2.2 and those expressing Olig2. On average, Nkx2.2 is expressed by cells that contain higher amounts of Gli activator in the nucleus, compared to cells expressing ectopic Olig2. Gli3A^{LOW} s= 0.0128, Olig2^{+} s= 0.0425, Nkx2.2^{+} s= 0.0586. Student's t-test: **Gli3A^{LOW}-Olig2^{+} P= 9.467×10^{-13}, ***Olig2^{+}-Nkx2.2^{+} P= 0.0007.


A Pax7
Olig2Pax7
GFPOlig2Pax7
B Olig2
Nkx2.2Olig2
GFPNkx2.2Olig2
C FoxA2
Nkx2.2 FoxA2
GFPNkx2.2FoxA2
Gli3A HIGH
D Olig2
Pax7 Olig2
Pax7 Olig2 GFP
E Isl1
Chx10 Isl1
GFP Chx10 Isl1
Gli3A MED
F Chx10
En1 Chx10
GFP En1 Chx10
Gli3A LOW
G
Level of Gli3A ΔN2
myc intensity/ pixel
0.12
0.1
0.08
0.06
0.04
0.02
0
*
**
Gli3A LOW
Olig2 +
Nkx2.2 +
Gli3A HIGH

3.5 Temporal integration of Shh/Gli signalling patterns the ventral neural tube.

The importance of signal duration as well as signal strength has been proposed to be an important parameter in morphogen signalling (Ahn and Joyner, 2004; Gurdon and Bourillot, 2001; Harfe et al., 2004). We therefore considered whether the integration of Shh/Gli activity over time might influence ventral neural tube patterning. We reasoned that if duration of signalling is critical, increasing the time of exposure to an activating signal would result in the induction of markers of increasingly more ventral domains, conversely, decreasing the time of exposure to a signal would have the opposite effect. To test this idea we focused on the induction of Nkx2.2 and Olig2, representative markers of Region A and Region B and as above we focused on forelimb and anterior thoracic levels. We first examined the time course of induction of these markers in Gli3A^{HIGH} transfected embryos. Transfection of Gli3A^{HIGH} was sufficient to induce Nkx6, Olig2, Nkx2.2 and FoxA2 at 12hpt, however, at 6hpt ectopic Nkx6 and Olig2 induction was evident but few if any transfected cells expressed Nkx2.2 or FoxA2 (Fig. 17A-G). Next we examined the induction of Nkx6, Olig2, Nkx2.2 and FoxA2 in embryos transfected with Gli3A^{MED}, robust induction of Nkx6 and Olig2 was evident 48hpt however few if any transfected cells ectopically expressed Nkx2.2 (Fig. 18A-D, G). In contrast, ectopic induction of Nkx2.2 and FoxA2 was evident in embryos transfected with Gli3A^{MED} 72hpt (Fig. 18E-G). We considered the possibility that these responses were the result of increased accumulation of activated Gli protein in transfected cells at later time points, however, measurements of protein levels in individual electroporated cells indicated that the amounts of Gli3A^{HIGH} and Gli3A^{MED} decreased over the time course of the experiment (Fig. 17H and 18H, green bars). Moreover, *in vivo* luciferase assays with GBS-Luc indicated that although the level of activity increased between 6hpt and 12hpt in Gli3A^{HIGH} transfected embryos (Fig. 17H,

blue bars), in embryos electroporated with Gli3A^{MED} the level of Gli activity did not increase between 24hpt and 72hpt (Fig. 18H, blue bars). Together these data suggest that the duration of activated Gli signalling is critical parameter determining the response of ventral neural tube cells.

We next asked if the duration of Shh signalling influenced patterning in a similar manner to the duration of activated Gli exposure. To test this we ectopically activated Shh signalling in neural tube cells for different lengths of time using SmoM2. At 12hpt cells electroporated with SmoM2 expressed Nkx6 and Olig2, however, few if any expressed Nkx2.2 or FoxA2 (Fig. 19A-D, G). In contrast by 24hpt ectopic Nkx2.2 and FoxA2 expressing cells were evident throughout the neural tube in SmoM2 transfectants (Fig. 19E-G). Assays with GBS-Luc indicated that the level of Gli activity in SmoM2 transfected embryos was similar at 12hpt and 24hpt (Fig. 19H, blue bars) suggesting that transfected cells were integrating the level of signalling over time rather than responding to increasing thresholds of Shh/Gli activity.

Our data support the idea that cells integrate Shh signalling over time and that the dorsal-ventral identity of a cell depends on a combination of the strength and duration of signal to which it is exposed. This would predict that the induction of Region B and Region A should proceed sequentially *in vivo*. We therefore examined the profile of Nkx2.2 and Olig2 induction in chick embryos. At somite level 12-14 in HH st12 (16som) embryos, Olig2 was detected in a domain of cells that encompassed the ventral midline (Fig. 20A). Low levels of Nkx2.2 were detected in a small number of the ventral-most cells adjacent to the underlying notochord (Fig. 20A). In slightly older embryos HH st12 (18som) regions level with somite 12-14, robust Nkx2.2 expression was induced in cells of the ventral midline (Fig. 20B) and Olig2 expression was observed in a broader ventral

domain with lower levels at the ventral midline (Fig. 20B). In HH st13 embryos (20som) at somite level 12-14 the more familiar pattern of Nkx2.2 and Olig2 expression began to resolve: Nkx2.2 was found in a ventral domain excluding the ventral midline (Fig. 20C) and dorsal to Nkx2.2 expressing cells Olig2 was expressed (Fig. 20C). This dynamic pattern of Nkx2.2 and Olig2 expression is consistent with the idea that individual progenitor cells are progressively ventralized and the mature expression domains are the result of the temporal integration of signalling during the early stages of neural tube patterning.

Figure 17. Sequential induction of ventral progenitors by Gli3A^{HIGH}.

HH st11–12 embryos were electroporated *in ovo* with Gli3A^{HIGH} and assayed 6h or 12h after electroporation (hpt) for the induction of the indicated ventral progenitor markers.

(a-f) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-F) showing expression of the markers.

(A-D) Gli3A^{HIGH} is sufficient to induce Olig2 (A) and Nkx6 (B) 6hpt but few cells upregulate Nkx2.2 (C) or FoxA2 (D).

(E, F) Nkx2.2 (E) and FoxA2 (F) are induced by Gli3A^{HIGH} at 12hpt.

(G) Quantitation of Olig2 and Nkx2.2 induction by Gli3A^{HIGH} at 6hpt and 12hpt.

(H) Graph shows the GBS-Luc Luciferase activity (blue bars) and the Gli3$^{\Delta N2}$ protein levels (green bars) at the indicated time points after electroporation with Gli3A^{HIGH}.

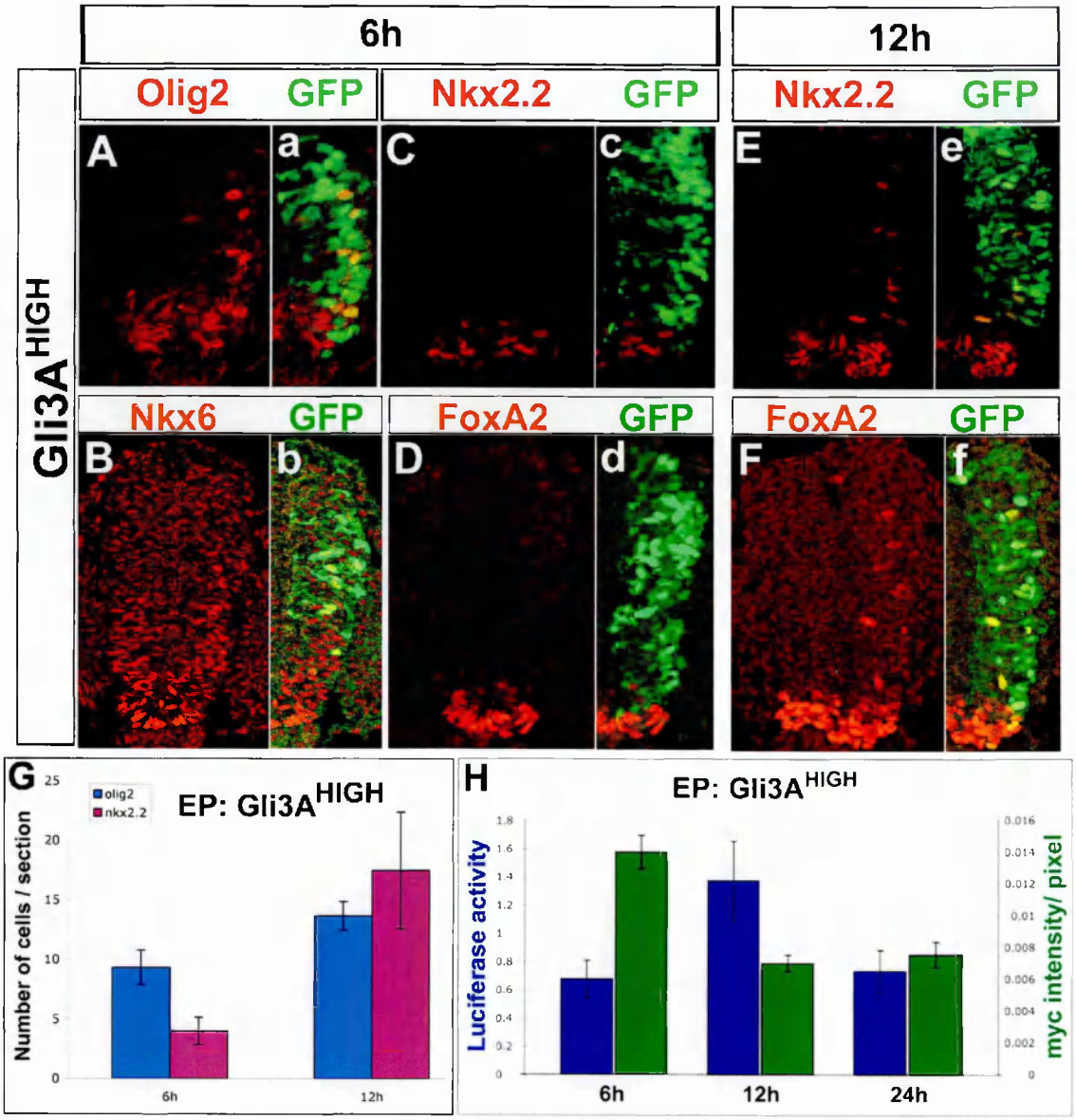

6h
12h
Olig2
GFP
Nkx2.2
GFP
Nkx2.2
GFP
Gli3A^HIGH
A
a
C
c
E
e
Nkx6
GFP
FoxA2
GFP
FoxA2
GFP
B
b
D
d
F
f
G
EP: Gli3A^HIGH
olig2
nkx2.2
Number of cells / section
25
20
15
10
5
0
6h
12h
H
EP: Gli3A^HIGH
Luciferase activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
myc intensity/ pixel
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
6h
12h
24h

Figure 18. Sequential induction of ventral progenitors by Gli3A^{MED}.

HH st11–12 embryos were electroporated *in ovo* with Gli3A^{MED} and assayed 48h or 72h after electroporation (hpt) for the induction of the indicated ventral progenitor markers.

(a-f) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-F) showing expression of the markers.

(A-D) Olig2 (A) and Nkx6 (B) but not Nkx2.2 (C) or FoxA2 (D) are upregulated by Gli3A^{MED} electroporated cells 48hpt.

(E, F) Gli3A^{MED} is sufficient to induce Nkx2.2 (E) and FoxA2 (F) by 72hpt.

(G) Quantitation of Olig2 and Nkx2.2 induction by Gli3A^{MED} at 48hpt and 72hpt.

(H) Graph indicating GBS-Luc Luciferase (blue bars) activity and the Gli3$^{\Delta N1}$ protein levels (green bars) at the indicated time points after electroporation with Gli3A^{MED}.

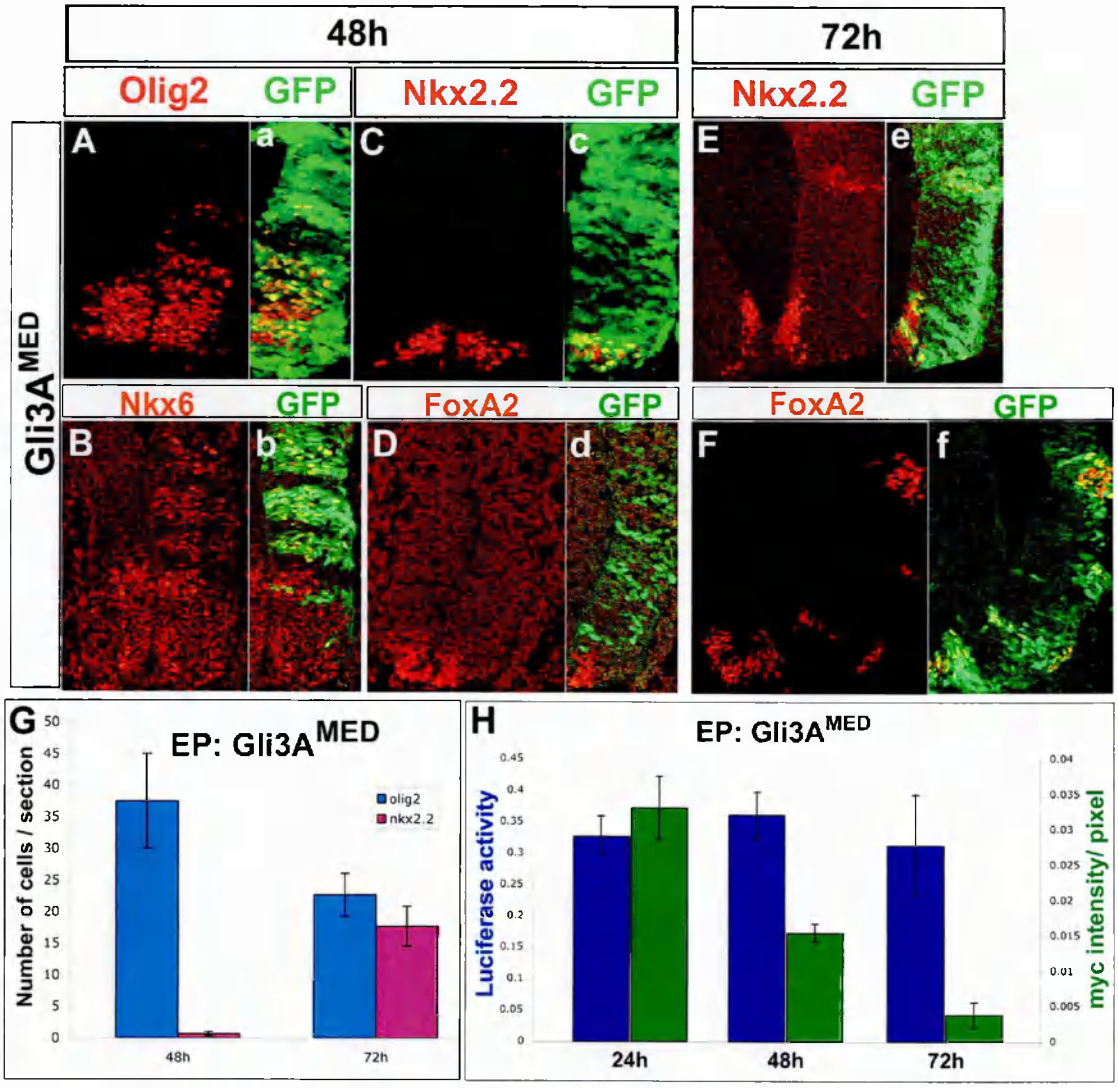

48h
72h
Olig2
GFP
Nkx2.2
GFP
Nkx2.2
GFP
Gli3A^MED
A
a
C
c
E
e
Nkx6
GFP
FoxA2
GFP
FoxA2
GFP
B
b
D
d
F
f
G
EP: Gli3A^MED
Number of cells / section
olig2
nkx2.2
48h
72h
H
EP: Gli3A^MED
Luciferase activity
myc intensity/ pixel
24h
48h
72h

Figure 19. Sequential induction of ventral progenitors by SmoM2.

HH st11–12 embryos were electroporated *in ovo* with SmoM2 and assayed 12h or 24h after electroporation (hpt) for the induction of the indicated ventral progenitor markers.

(a-f) Overlay of green channel (GFP), indicating electroporated regions of the neural tube, with red channel (A-F) showing expression of the markers.

(A-D) Olig2 (A) and Nkx6 (B) are upregulated by SmoM2 transfected cells 12hpt. At this time point Nkx2.2 (C) is induced by few cells and FoxA2 (D) is not induced by SmoM2 transfected cells.

(E, F) SmoM2 is sufficient to induce Nkx2.2 (E) and FoxA2 (F) by 24hpt.

(G) Quantitation of Olig2 and Nkx2.2 induction by SmoM2 at 12hpt and 24hpt.

(H) Graph shows the GBS-Luc Luciferase activity at the indicated time points after electroporation with SmoM2.

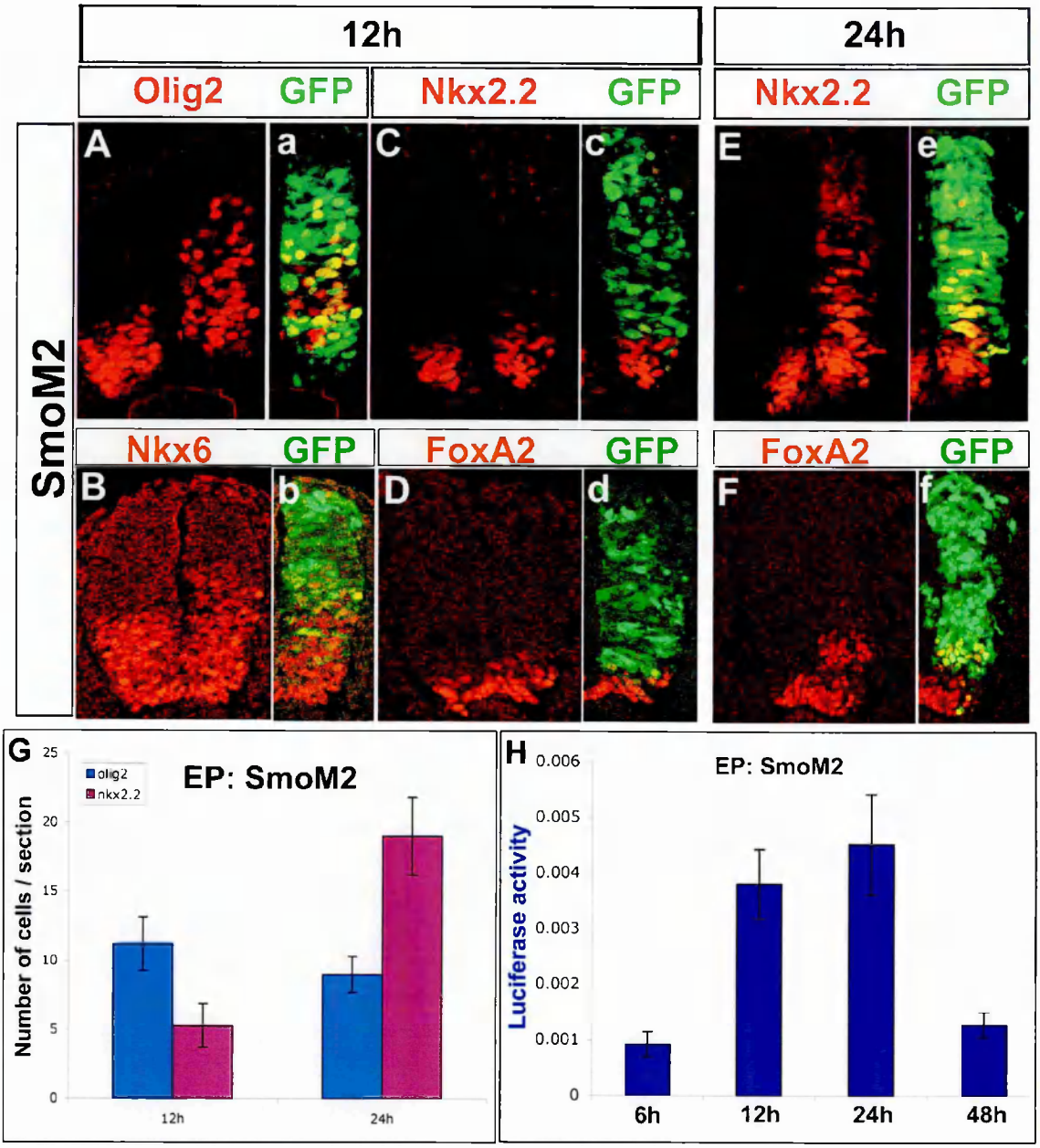
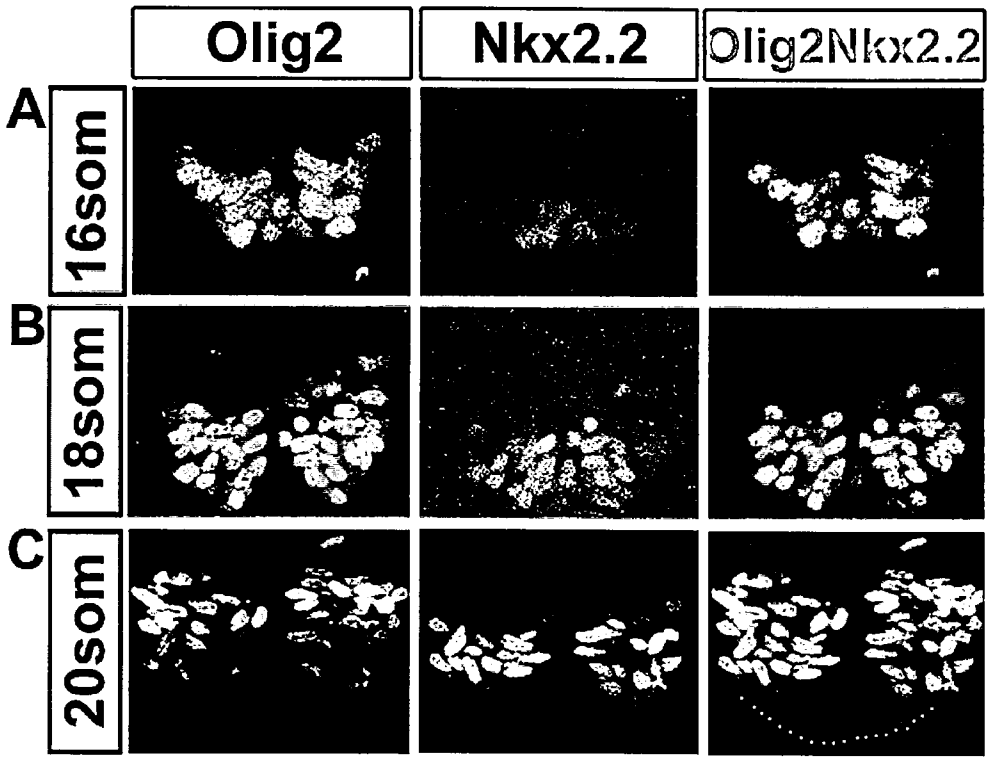

12h
24h
Olig2
GFP
Nkx2.2
GFP
Nkx2.2
GFP
SmoM2
A
a
C
c
E
e
Nkx6
GFP
FoxA2
GFP
FoxA2
GFP
B
b
D
d
F
f
G
EP: SmoM2
olig2
nkx2.2
Number of cells / section
25
20
15
10
5
0
12h
24h
H
EP: SmoM2
Luciferase activity
0.006
0.005
0.004
0.003
0.002
0.001
0
6h
12h
24h
48h

Figure 20. Sequential induction of the endogenous ventral progenitors.

(A) In early HH st12 chick embryo (16 somites) at the cervical level, Olig2 is expressed in the ventral neural tube, including the ventral midline. Low Nkx2.2 expression is detected in a small number of cells at the ventral midline.

(B) In late HH st12 chick embryo (18 somites) at the cervical level, Olig2 is expressed in a broader ventral domain and downregulated at the ventral midline. Nkx2.2 is induced at the ventral midline.

(C) In a HH st13 chick embryo (20somites) at the cervical level, Nkx2.2 is expressed ventrally, excluding the ventral midline. Olig2 is expressed dorsal to Nkx2.2.


Olig2
Nkx2.2
Olig2Nkx2.2
A
16som
B
18som
C
20som

3.6 Alternative approaches to analyze graded Gli activity.

3.6.1 Analysis of Gli3-ZF-VP16$_{(4)}$ as Gli activator candidate.

One candidate construct that we generated and assayed for Gli activator function was Gli3-ZF-VP16$_{(4)}$. Gli3-ZF-VP16$_{(4)}$ comprises of the DNA binding domain (zinc fingers) of Gli3 fused to four repeats of the VP16 transactivation domain (Ohashi et al., 1994). As shown earlier a Gli activator is expected to increase levels of activity from GBS-Luc reporter construct and to ventralize the intermediate and dorsal neural tube, repressing class I HD proteins and inducing class II HD proteins.

In vitro Gli3-ZF-VP16$_{(4)}$ functioned as a strong activator of transcription, increasing the FF Luciferase activity approximately 30 times compared to control (Fig. 7B). In the neural tube of chick embryos electroporated with Gli3-ZF-VP16$_{(4)}$ the prediction was that class I HD proteins would be repressed and class II HD proteins would be ectopically induced in the intermediate and dorsal neural tube, instead all the progenitor markers we assayed were repressed. Both class I proteins Pax7 (Fig. 21B), Pax6 (Fig. 21C) and class II proteins Nkx6.1 (Fig. 21D) and Nkx2.2 (Fig. 21E) were downregulated in the Gli3-ZF-VP16$_{(4)}$ transfected cells. Moreover, Gli3-ZF-VP16$_{(4)}$ expressing cells were frequently localized laterally, in a position characteristic of postmitotic neurons, however, neuronal markers Cyn1, HB9/MNR2 and Tuj1 (Fig. 22B-D) were also downregulated. In addition, it was evident in a number of transfected embryos that electroporated cells were migrating away from the neural tube, a situation not encountered in control transfections. We hypothesized that Gli3-ZF-VP16$_{(4)}$ might induce neural crest formation and this could account for the observations. To test this possibility we examined expression of the neural crest marker HNK1. In some neural tubes we observed intense HNK1 staining (Fig. 22E) within the neural tube; much of this expression was in cells not transfected with Gli3-ZF-VP16$_{(4)}$. Thus, Gli3-ZF-VP16$_{(4)}$

either upregulates HNK1 expression non-autonomously or HNK1 expressing cells occupy the region of the neural tube vacated by migrating Gli3-ZF-VP16$_{(4)}$ transfected cells. In addition, in many cases laminin staining which marks the basal membrane (Fig. 22F) indicated that the structure of the Gli3-ZF-VP16$_{(4)}$ electroporated neural tubes was disrupted with many of the electroporated cells being displaced into the somites.

Together these data suggest that Gli3ZnF-VP16$_{(4)}$ induces the expression of genes not normally induced by Shh signalling or Gli proteins and this results in the delamination and/or migration of neural cells. We are investigating this possibility further.

3.6.2 Express a concentration gradient of a Gli activator along the anterior-posterior (AP) axis of transgenic mouse embryos.

A fragment of chick Hoxa7 promoter drives graded expression in the neural tube, forming a posterior to anterior gradient (Gaunt, 2001). We wished to generate transgenic mice expressing Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ under Hoxa7 promoter control and test if at different positions along the AP axis different ventral neuronal subtypes were induced. Our hypothesis predicted that in the posterior neural tube the most ventral cell types would be generated (floor plate, V3 neurons) while MNs, V2, V1 neurons would be generated in more rostral regions.

Hoxa7 promoter sequence containing a short fragment of the coding sequence of HoxA7 was fused to nuclear targeted GFP sequence. Electroporation of this construct in chick embryos resulted in GFP expression in the neural tube 24hpt and 48hpt (Fig. 23A,B). In the majority of the electroporated embryos 24hpt the intensity of GFP fluorescence was higher in caudal regions (eg. hindlimb level) compared to more rostral regions (eg. forelimb level), compare Fig. 23A1 and 23A3.

The following sequences were cloned/inserted downstream of the GFP coding sequence: IRES in order to facilitate bicistronic expression; either Gli3A$^{\Delta N1}$ or Gli3A$^{\Delta N2}$ coding sequences; SV40 polyadenylation signal to provide stability of the transcript (see materials and methods for cloning information). The targeting constructs were named Hoxa7-Gli3A$^{\Delta N1}$ and Hoxa7-Gli3A$^{\Delta N2}$ based on the Gli activator they contained. We tested the function of Hoxa7-Gli3$^{\Delta N1}$ by electroporating chick embryos. 48hpt GFP expression was detected at forelimb, thoracic and hindlimb regions along the AP axis of the spinal cord. At the thoracic and hindlimb levels HB9/MNR2 was induced in ectopic regions while at the forelimb level GATA3 was expressed by cells dorsal to the normal boundary of GATA3 expression, indicating the generation of ectopic MNs and V2 neurons respectively (Fig. 23C-E). These data show that, at least in the chick electroporation assay, Hoxa7-Gli3A$^{\Delta N1}$ sequence maintains the ability of Gli3$^{\Delta N1}$ to induce Region B cell fates.

Hoxa7-Gli3$^{\Delta N1}$ and Hoxa7-Gli3$^{\Delta N2}$ constructs were linearized and pronuclear injections performed in order to generate transgenic mouse embryos. Out of 20 Hoxa7-Gli3$^{\Delta N1}$ and 104 Hoxa7-Gli3$^{\Delta N2}$ injected embryos no GFP expressing or positive for myc immuno-reactivity or with ectopic MNs were identified. Embryos were analysed at E10.5.

Figure 21. Gli3-ZF-VP16$_{(4)}$ represses both class I and class II HD proteins.

HH stage 10 – 12 embryos were electroporated *in ovo* with Gli3-ZF-VP16$_{(4)}$ and assayed 48h later for the expression of the indicated genes.

(A) Diagram summarizing the Gli3-ZF-VP16$_{(4)}$ construct. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; VP16, VP16 transactivation domain.

(B-E) Repression of Pax7 (B; red), Pax6 (C; red), Nkx6 (D; red) and Nkx2.2 (E; red) in the neural tube of embryos electroporated with Gli3-ZF-VP16$_{(4)}$ (green).

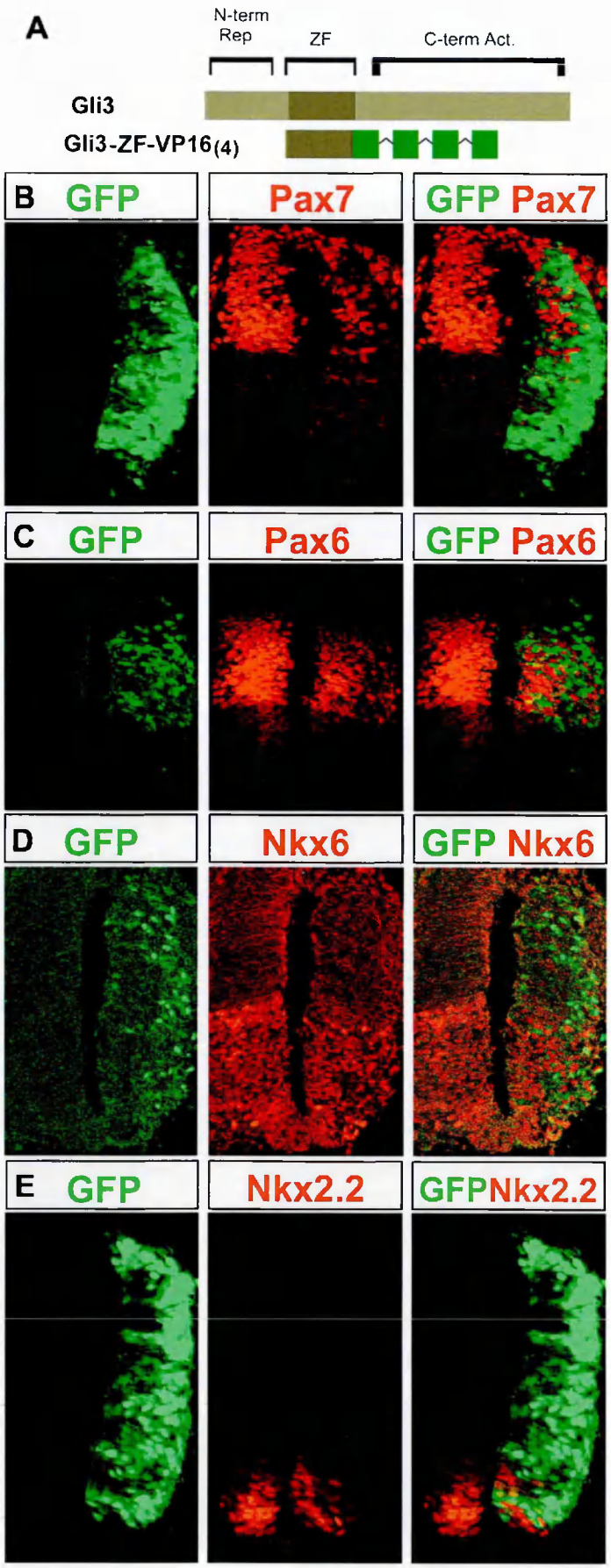

A
N-term Rep
ZF
C-term Act.
Gli3
Gli3-ZF-VP16(4)
B GFP
Pax7
GFP Pax7
C GFP
Pax6
GFP Pax6
D GFP
Nkx6
GFP Nkx6
E GFP
Nkx2.2
GFPNkx2.2

Figure 22. Gli3-ZF-VP16$_{(4)}$ disrupts neural tube structure.

HH stage 10 – 12 embryos were electroporated *in ovo* with Gli3-ZF-VP16$_{(4)}$ and assayed 48-72h later for the expression of the indicated genes.

(A) Diagram summarizing the Gli3-ZF-VP16$_{(4)}$ construct. ZF, zinc finger DNA binding domain; N-term Rep, region of Gli3 amino terminal of the DNA binding domain implicated in repressing transcription; C-term Act, putative transcriptional activation domain carboxy-terminal of the zinc finger domain; VP16, VP16 transactivation domain.

(B, C) Cyn1^{+} (B; red) and MNR2/HB9^{+} (C; red) neurons are reduced in Gli3-ZF-VP16$_{(4)}$ (green) electroporated embryos.

(D) Post-mitotic neurons Tuj1 (red) are reduced and are not arranged laterally in the Gli3-ZF-VP16$_{(4)}$ electroporated side of the neural tube. Many transfected cells (green) migrate out of the neural tube.

(E) Neural crest marker HNK1 (red) is either upregulated in the Gli3-ZF-VP16$_{(4)}$ (green) electroporated side of the neural tube in a cell non-autonomous manner, or neural crest cells invade the electroporated side of the neural tube.

(F) Laminin staining (red) shows that the pial surface of the the Gli3-ZF-VP16$_{(4)}$ (green) electroporated side of the neural tube is disrupted.

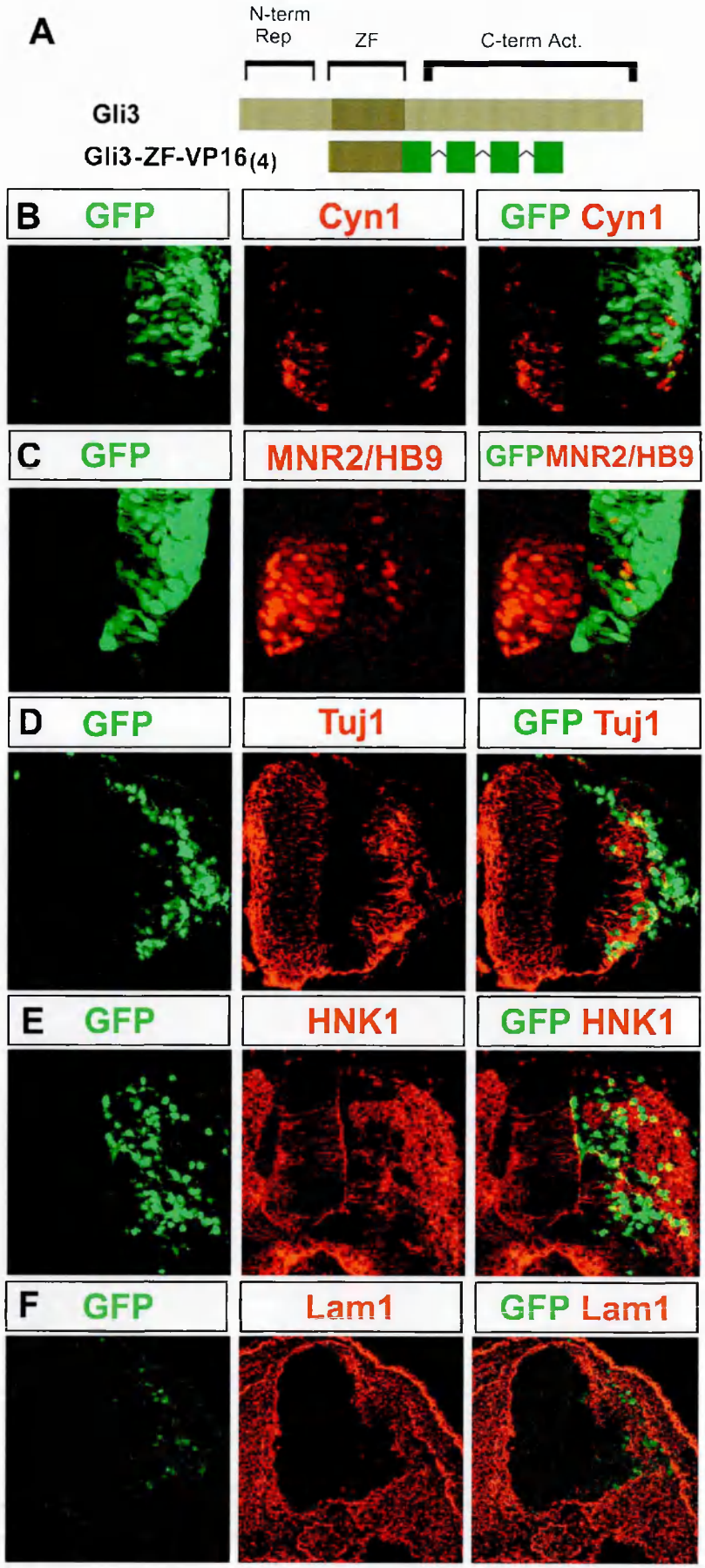
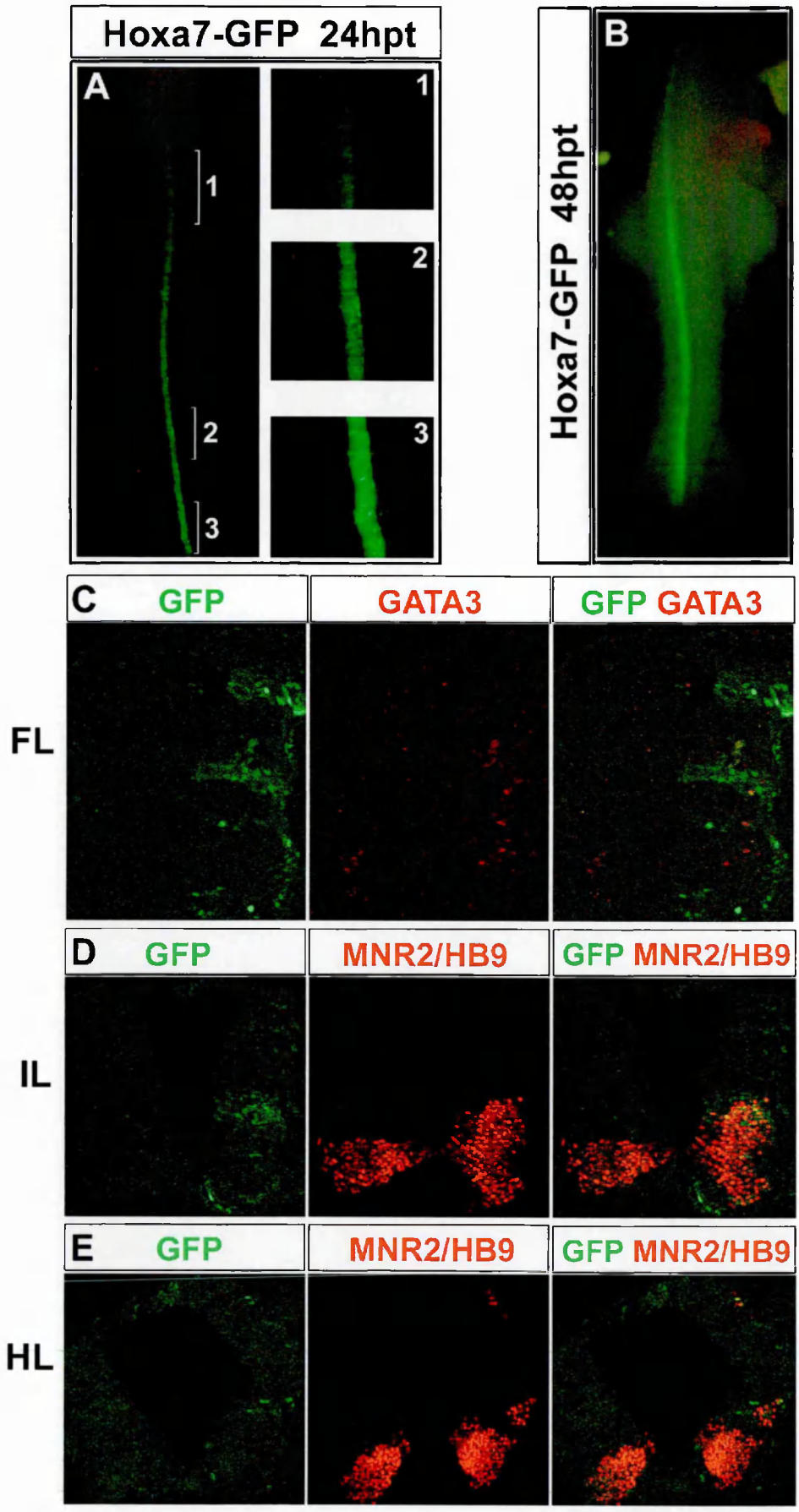

A
N-term Rep
ZF
C-term Act.
Gli3
Gli3-ZF-VP16(4)
B GFP
Cyn1
GFP Cyn1
C GFP
MNR2/HB9
GFPMNR2/HB9
D GFP
Tuj1
GFP Tuj1
E GFP
HNK1
GFP HNK1
F GFP
Lam1
GFP Lam1

Figure 23. Hoxa7-GFP construct seems to express in a graded manner from posterior to anterior in the chick neural tube and expresses levels of Gli3A^{MED} sufficient to induce V2 neurons and MNs.

(A, B) HH stage 10 – 12 embryos were electroporated *in ovo* with Hoxa7-GFP and GFP expression detected with epifluorescence 24h or 48h later. At both time points GFP expression was higher in caudal regions of the embryo (eg A2; posterior interlimb area) compared to rostral regions (eg A1; cervical level)

(C-E) HH stage 10 – 12 embryos were electroporated *in ovo* with Hoxa7-GFP-Gli3A$^{\Delta N1}$ and assayed 48hpt for expression of GATA3 (C), MNR2/HB9 (D, E). FL, forelimb level; IL, interlimb level; HL, hindlimb level.


Hoxa7-GFP 24hpt
A
1
2
3
1
2
3
Hoxa7-GFP 48hpt
B
C
GFP
GATA3
GFP GATA3
FL
D
GFP
MNR2/HB9
GFP MNR2/HB9
IL
E
GFP
MNR2/HB9
GFP MNR2/HB9
HL

4. DISCUSSION

The ability of graded Shh signalling to organize cellular pattern serves as a paradigm for vertebrate morphogen gradients. Shh signalling is sufficient to control cell fate along the dorsal-ventral axis of the neural tube and a range of evidence indicates that Shh functions at a long range to elicit distinct outputs at different concentration thresholds (Briscoe et al., 2001; Ericson et al., 1997a; Gritli-Linde et al., 2001; Hynes et al., 2000; Lewis et al., 2001; Matise et al., 1998; Wijgerde et al., 2002). Major interest resides, therefore, in understanding the mechanisms by which Shh signalling controls gene expression and cell patterning. In this study we have examined the role that Gli mediated transcription plays in Shh signalling. First, using two dominant repressor versions of Gli, we demonstrate that blocking Gli mediated transcription prevents ventral neural tube patterning and the generation of the appropriate neuronal subtypes resulting in the dorsalisation of the ventral neural tube. Second, we demonstrate that Gli transcriptional activity is sufficient to mediate Shh responses and an activity and temporal gradient of Gli mediated transcription is sufficient to emulate graded Shh signalling. We provide evidence that even in the absence of ongoing Shh signalling Gli activity is able to mediate Shh patterning effects in the ventral neural tube. Moreover, our analysis indicates that the 2-3 fold incremental changes in Shh concentration that generate alternative neuronal subtypes in vitro can be mimicked by similar changes in the level of Gli activity *in vivo*. This suggests that the Shh signalling pathway does not substantially amplify signal differences during transduction to the nucleus. Instead, modest differences in the amount or duration of Gli activity are able to differentially control gene expression. Together, these data suggest that graded Shh signalling is translated into a gradient of Gli activity and that small, cell autonomous changes in the level of Gli activity is sufficient to orchestrate ventral neural tube patterning (Fig. 24).

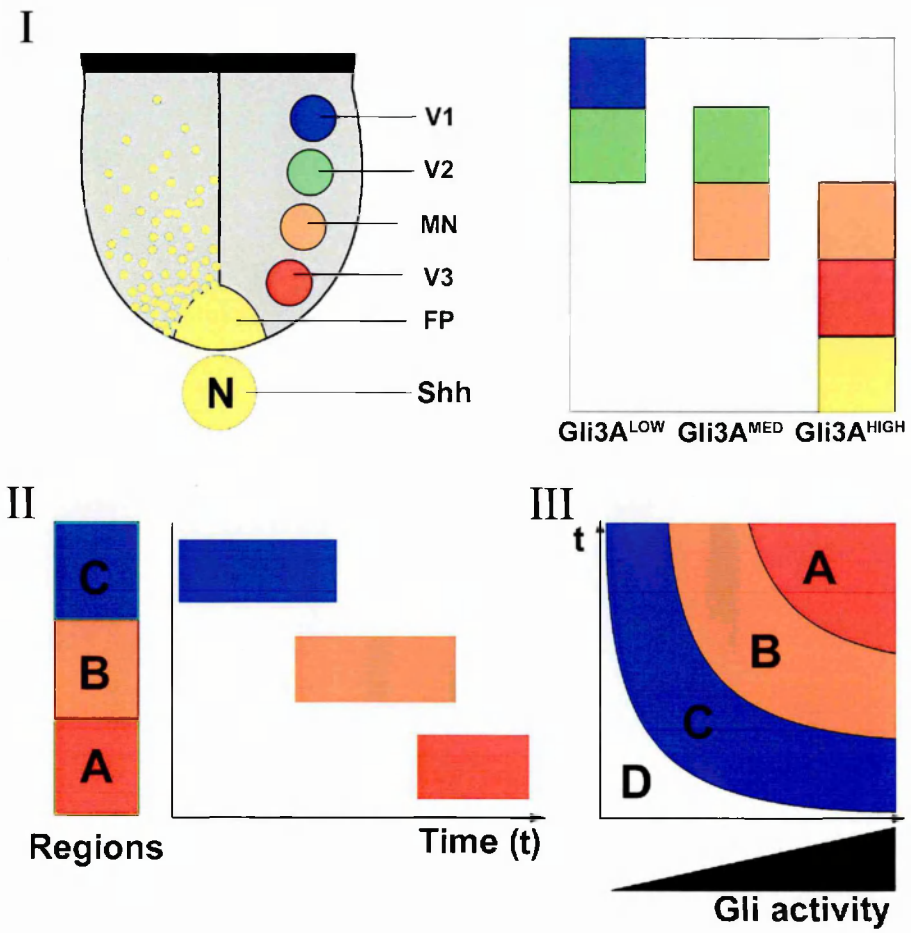


Figure 24. A model of Gli function in ventral neural tube patterning

(I) A gradient of Gli transcriptional activity emulates graded Shh signalling and is sufficient to orchestrate patterning of the ventral neural tube.

(II) In addition to signal strength, signal duration determines dorsal-ventral patterning. Exposure to a fixed level of Shh signalling for an increasing time results in the incremental induction of more ventral markers.

(III) Levels of Shh/Gli activity are integrated over time thus both signal strength and signal duration contribute to the patterning of the neural tube.

4.1 An essential role for Gli activity in dorsal-ventral patterning of the spinal cord.

Embryos lacking individual Gli proteins have limited dorsal-ventral patterning defects. The most ventral region of the neural tube (floor plate and V3 neurons) is markedly reduced in embryos lacking Gli2 (Ding et al., 1998; Matise et al., 1998) and the patterning of the intermediate spinal cord is disrupted in embryos lacking Gli3 (Persson et al., 2002), but MNs and V2 neurons are still present in both mutants. It is possible that MNs and V2 neurons are generated by an entirely Gli independent mechanism, however, the forced expression of two dominant Gli repressor proteins, Gli3R and Gli3-ZF-EnR indicates the generation of these cell types can be blocked by inhibiting Gli mediated transcription. These data suggest a central role for Gli mediated transcriptional control in the generation of ventral neurons. The limited defects seen in individual knock out animals argue for functional redundancy between Gli proteins. Consistent with this idea Gli2 and Gli3 have been proposed to have redundant functions during lung, skeletal and tooth development (Hardcastle et al., 1998; Mo et al., 1997; Motoyama et al., 1998). Moreover, Gli2 and Gli3 can function as both repressors and activators of transcription and Gli binding sites confer responsiveness to each of the Gli proteins, *in vitro* (Dai et al., 1999; Sasaki et al., 1999).

In mouse embryos lacking all Gli protein function MNs, V0, V1 and V2 neurons are generated but their spatial organization is severely disrupted and the different neuronal subtypes are generated in an apparently stochastic manner, irrespective of their position along the dorsal ventral axis (Bai et al., 2004; Lei et al., 2004). The generation of neuronal types with ventral identities in the *Gli* null mouse embryos could be the result of derepression of Shh target genes similar to the case with the *Drosophila* homologue Ci. In the absence of Hh/ Shh signal Gli2 and Gli3 are proteolytically cleaved to form transcriptional repressors (Aza-Blanc et al., 2000; Dai et al., 1999; Wang et al., 2000),

consequently, *Gli* null mice abolish both the Gli transcriptional activator function, which reflects the presence of Shh, and the transcriptional repressor, which is produced in the absence of Shh. In this view, the expression of ventral progenitor markers in the absence of all Gli proteins could be the result of removing the transcriptional repression that Gli2 and Gli3 normally exert in the absence of Shh signal. Consistent with this Methot and Basler suggested that the phenotypes observed in the absence of Ci are mild compared with the Hh mutants because in the absence of Hh the Hh target genes are actively repressed, a function missing in the absence of Ci (Methot and Basler, 2001). Accordingly, if a gene can be expressed independently of Gli activity this doesn't mean it is insensitive to regulation by Gli proteins.

The intermingling and incorrect positioning of the ventral progenitors and neurons in mouse embryos lacking all Gli activity indicates that the function of Gli proteins is necessary for generation of the ventral neuronal types in the appropriate positions along the dorsal ventral axis of the neural tube. Taken together the effects of dominant negative Gli and the phenotype of the *Gli* null mice suggest that Gli proteins can exert transcriptional control over the generation of ventral progenitors and neuronal subtypes and the Gli proteins are necessary for the spatial organization of the ventral neural tube.

Electroporation of the dominant repressor Gli3-ZF-EnR results in the dorsalisation of the ventral neural tube when expressed at low levels, however, expression of Gli3-ZF-EnR at high levels resulted in a large increase in apoptosis. This is consistent with observations that Ptc1, which blocks Shh signalling, inhibits ventral patterning, affecting the proliferation rate of cells (Barnes et al., 2001) and can cause apoptosis (Thibert et al., 2003). Together these data suggest that high levels of Gli mediated repression completely blocks the Shh pathway, leading to cell death, while lower levels of Gli3-ZF-

EnR (moderate repression) are sufficient to block patterning of Shh without affecting cell survival.

4.2 A gradient of Gli activity is sufficient to mediate graded Shh signalling.

Although the data suggest that Gli function is required for the correct spatial patterning of the ventral spinal cord, they raise the question of whether Gli transcriptional activity is sufficient to mediate Shh signalling responses. We have addressed this question, using two amino-terminal deletions of human Gli3 that work as constitutive activators of Shh signalling pathway. We show that dominant Gli activity upregulates, *in vivo*, the expression of *Ptc1, Ptc2* and *Gli1,* genes considered as molecular readouts of Shh signalling (Pearse et al., 2001). In addition Gli activity induces the formation of ventral neuronal subtypes dorsal to their normal boundaries and results in the ventralisation of the dorsal neural tube. Consistent with our data, mouse embryos expressing an amino-terminal deletion of Gli2 or Gli3 (Sasaki et al., 1999) ectopically express FoxA2 and Isl1 in the dorsal midbrain/hindbrain, suggesting that activating versions of the Gli proteins can induce floor plate cells and MNs. In addition more recent data from Lei et al 2004 show that amino-terminal deletions of Gli2 and Gli3 can upregulate the expression of Shh target genes in the neural tube. Together these results indicate that Gli activity is sufficient to induce Shh responses and direct patterning of the ventral neural tube.

The principal characteristic of a morphogen gradient is that cells activate and/or repress different sets of genes at different concentration thresholds. In the case of Shh signalling, loss of function and gain of function experiments suggest that Gli activity is necessary and sufficient to mediate Shh signalling responses. This raises the question of whether Gli mediated transcription is sufficient to recapitulate graded Shh signalling and how Gli proteins provide this positional information to the developing neural tube. Our data

indicate that the full repertoire of ventral identities can be generated with different levels of Gli activity. Moreover, the level of Gli activity necessary to generate a particular cell fate corresponds to its position of generation *in vivo*. Therefore these data indicate that Gli dependent transcription is sufficient to mediate Shh signalling and suggest that the extracellular concentration gradient of Shh is translated intracellularly into a gradient of Gli activity which patterns the ventral neural tube.

In vivo, pCAGGS-Gli3A$^{\Delta N2}$ consistently generates 2-3 fold higher GBS-Luc reporter activity than pCAGGS-Gli3A$^{\Delta N1}$ electroporated neural cells (Fig. 9C), however, when assayed *in vitro*, in C3H/10T1/2 cells, pCAGGS-Gli3A$^{\Delta N1}$ and pCAGGS-Gli3A$^{\Delta N2}$ generated similar levels of activity (Fig. 7B). When transfected in a different cell line, P19 cells (McBurney et al., 1982), pCAGGS-Gli3A$^{\Delta N2}$ produced 2-3 fold higher activity compared to pCAGGS-Gli3A$^{\Delta N1}$ (F. Ulloa and J. Briscoe, unpublished results). These observations indicate the importance of the cell context that this assay is performed and suggest that a modulator or modulators of Gli activity, present in the embryos and in P19 cells are missing from C3H/10T1/2 cells.

If graded Gli activity is sufficient to mediate Shh signalling does a gradient of Gli activity exist *in vivo*? An indication of the presence of a Gli activity gradient is that Gli1 expression is higher in the ventral neural tube and lower in the intermediate neural tube (Marigo et al., 1996b). Moreover, data from electroporation of HH st 11-12 chick embryos with a reporter construct containing Gli binding sites and GFP as a reporter gene show that there is a correlation between the distance of a cell from the ventral midline/ Shh source and its intensity of GFP fluorescence (F. Ulloa, J. Briscoe, unpublished data). These data suggest that endogenous Gli proteins generate an activity gradient, *in vivo*.

If the endogenous Gli proteins form an activity gradient how is this gradient constructed from the endogenous Gli proteins? Addressing this question is complicated by the presence of three Gli proteins with different expression profiles in the neural tube, which have been assigned unique as well as overlapping functions (Aza-Blanc et al., 2000; Dai et al., 1999; Hui et al., 1994; Ingham and McMahon, 2001; Jacob and Briscoe, 2003; Lee et al., 1997; Marigo et al., 1996b; Ruiz i Altaba, 1998; Ruiz i Altaba et al., 2003; Sasaki et al., 1997; Wang et al., 2000). Taken together with the findings presented in this study, the data suggest that the aggregate of individual responses of each Gli protein to a given concentration of Shh results in a gradient of Gli activity in the neural tube. In this view, in the absence of Shh signalling, Gli repressor activity dominates; low levels of Shh signalling would inhibit Gli repressor formation facilitating the production of Region C. At higher levels of Shh signalling, repressor activity would be absent and it seems likely that a combination of Gli2 and Gli3 activators direct the generation of Region B. At the highest levels of Shh signalling, *Gli3* expression is repressed and the production of Gli2 activator isoforms induces *Gli1* expression and the generation of Region A. This model also provides an explanation for the different neural patterning defects observed in mouse and zebrafish embryos lacking the same *Gli* orthologue (Karlstrom et al., 2003): small differences in the activity and/or response of the orthologues in the two species result in different profiles of functional overlap and redundancy. Consequently, lack of an orthologue in mouse and zebrafish has different effects on the resulting gradient of Gli activity.

4.3 How is a gradient of Shh signalling converted into all or none changes in gene expression?

The finding that the extracellular gradient of Shh can be represented by an intracellular gradient of Gli activity provides mechanistic insight into the perception of a morphogen gradient, it does not however, address how discrete, all or none changes in gene expression are generated at distinct ligand concentrations. Class I and II homeodomain proteins expressed by progenitors act to impose progenitor cell identity and cross-repressive interactions between these proteins, which are expressed in complementary domains, act to refine and maintain domains of expression (Briscoe and Ericson, 2001; Briscoe et al., 2000). In this view, graded Gli activity would establish initial asymmetries of homeodomain protein expression and then cross-repressive interactions between them would result in the abrupt changes in gene expression observed in the neural tube generating discrete progenitor domains.

This model resembles mechanisms involved in other developmental processes, notably the anterior-posterior patterning of the *Drosophila* embryo. Bicoid, the first protein shown to function as a morphogen, acts to pattern the anterior posterior axis of the *Drosophila* embryo (Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-Volhard, 1988b). Bicoid is also transcriptional activator and induces the expression of Gap genes e.g. *hunchback, Krüppel, knirps, giant* (reviewed by Rivera-Pomar and Jackle, 1996). In the case of *Krüppel*, in addition to its regulation by bicoid, *Krüppel* is repressed by Gap genes *knirps* and *giant*, which are expressed adjacent to the Krüppel domain (Ephrussi and St Johnston, 2004; Riechmann and Ephrussi, 2001; Rivera-Pomar and Jackle, 1996). Like class I and II proteins the Gap genes are expressed as a response to a signal gradient and there is cross regulation between Gap genes. It is possible therefore that the establishment of initial asymmetries of target genes expression, under

the influence of a graded signal, followed by cross-repressive interactions between the target genes, represents a general strategy for the provision of positional information and generation of thresholds during development.

4.4 Gli3 repressors and activators mediate Shh responses in a cell autonomous manner.

Electroporation of dominant Gli activators or repressors results in altered expression patterns of progenitor and neuronal markers only in the transfected cells showing that Gli proteins are sufficient to cause these alterations in gene expression and neural cell fates in a cell autonomous manner. Consistent with this when Shh signal was blocked at the membrane level with $Ptc1^{\Delta loop2}$ electroporation of chick embryos or in chimeric mouse embryos containing *Smo-/-* cells (Briscoe et al., 2001; Wijgerde et al., 2002) the dorsalization effect was also cell autonomous. In other developmental processes community effects have been observed. In these cases development of neighbouring progenitor cells is co-ordinated such that the identity of a cell depends on the identity of its neighbouring cells. Therefore a minimum number of cells and close contact between them is an important aspect of patterning (Gurdon et al., 1993; Standley et al., 2001). For example, Hox expression patterns can be autonomously maintained in ectopic locations within the hindbrain only if they remain in a group in transplantation experiments; the cells that become separated from the primary graft and intermingle with the surrounding populations loose the expression of the appropriate *Hox* genes (Trainor and Krumlauf, 2000). Evidence for a community effect has also emerged from studies of muscle cell differentiation in which there is a minimum number of *Xenopus* muscle progenitor cells required in order to undergo differentiation; cell aggregates of less that a hundred muscle progenitors cannot differentiate (Gurdon et al., 1993). The data presented here showing that expression of Gli proteins can change gene expression and the cell fate in single

cells, together with data from Ptc1$^{\Delta loop2}$ and *Smo-/-* studies demonstrate that cells can respond to changes in Shh signalling in a cell autonomous manner and argue against the existence of a community effect in Shh signalling responses in the neural tube.

Conversely, alterations in Shh signalling can cause non autonomous effects. Blocking Shh signalling with Ptc1$^{\Delta loop2}$ allows Shh to spread further from the midline than normal, since Ptc1$^{\Delta loop2}$ does not bind Shh, and results in the non autonomous induction of ventral markers as Nkx2.2 (Briscoe et al., 2001). Gli repressors and activators also cause non autonomous effects, as they change *Ptc1* expression and concomitantly spreading of Shh, resulting in non cell autonomous changes of the expression of Shh target genes. For example, Gli3R electroporation results in ventral boundary of Pax6 expression slightly shifted dorsal (Fig. 4F), while Gli3A^{HIGH} electroporation causes expansion of Pax7 expression ventral to its normal boundary (Fig. 11A).

Forced expression of dominant Gli activators can induce the full range of Shh responses. It is likely that some of these responses are the result of induction of ectopic Shh expression. Electroporation of Gli3A^{HIGH} construct induces ectopic floor plate cells (Fig. 10C) along the dorsal ventral axis of the neural tube and ectopic expression of Shh (Fig. 13D). Our data show that the patterning effects of Gli activators were not altered by blocking Shh signalling at the cell membrane level when co electroporated with HIP or Ptc1$^{\Delta loop2}$ (J. Briscoe, unpublished results), suggesting that Gli3A^{HIGH}, Gli3A^{MED} and Gli3A^{LOW} can recapitulate graded Shh responses even in the absence of ongoing Shh signalling. Our findings demonstrate that the dominant Gli activators exert their patterning function in a cell autonomous way and not through the induction of ectopic Shh expression.

4.5 Small changes in the level of Gli activity are sufficient to orchestrate dorsal-ventral patterning.

In vitro data indicate that incremental 2-3 fold changes in Shh concentration are sufficient to direct the generation of different cell types characteristic of the ventral neural tube (Ericson et al., 1997a). Two mechanisms can be envisioned by which these changes in the extracellular concentration of signal could be quantitatively transduced to the nucleus. First, differences could be amplified during intracellular signal transduction so that small extracellular differences result in substantial intracellular differences in transcriptional activity. Signalling pathways involving kinase cascades, such as the MAP kinase pathway, have been proposed to operate in this manner (Duffy and Perrimon, 1996). A second possibility is that the small differences in receptor activation could be maintained at each step inside the cell, so that the level of transcriptional activity directly reflects the extracellular concentration of the signal (Shimizu and Gurdon, 1999). Our data suggest Shh signalling adopts this latter strategy. The three dominant active Gli constructs used in this study differ in activity by increments of 2 – 3 fold, these are sufficient to recapitulate ventral neural tube patterning and simulate the effect of exposure of neural explants to 2 – 3 fold differences in Shh concentration (Ericson et al., 1997a). Consistent with this we find that Gli3A^{HIGH} expresses 4-6 fold more protein than Gli3A^{LOW} and these constructs generate gene expression profiles produced, *in vitro*, by similar differences in Shh concentration. Moreover, cells transfected with Gli3A^{HIGH} that express Nkx2.2 contain on average ~50% higher levels of Gli3A^{HIGH} than transfectants expressing Olig2. Consistent with this, production of Nkx2.2 expressing progenitors requires concentrations of Shh in excess of 3nM, while MN generation peaks at 2nM (Ericson et al., 1997a). Together the data support the idea that the 2-3 fold differences in extracellular Shh concentration are transduced by similar small differences in the level of

Gli activity and suggest that little if any amplification of the signal occurs during signal transduction.

Analyses of other morphogen signalling pathways have led to similar conclusions. Three fold changes in activin concentration, sufficient to switch between different cell fates in *Xenopus* animal cap explants, result in three fold changes in nuclear Smad2 suggesting that activin signal is transduced without amplification (Shimizu and Gurdon, 1999). In addition, in Toll-Dorsal signalling, a similar linear correlation between the activated Toll receptors and the nuclear Dorsal has been proposed since there are not multiple enzymatic steps along the transduction pathway (Belvin and Anderson, 1996; Stathopoulos and Levine, 2002b). Thus a common feature of graded signals appears to be that differences in signal strength are relayed directly, without amplification, to the nucleus. This may reflect the mechanism of signal transduction; Stathopoulos & Levine (2002) suggest that transduction pathways that include multiple enzymatic steps e.g. receptor tyrosine kinase pathways, are better suited to produce on/off responses to extracellular gradients while other pathways that do not amplify the signal significantly are probably better suited to generate multiple threshold responses.

4.6 Transcriptional regulation of Shh target genes

The demonstration that small changes in the level of Gli activity are sufficient to differentially regulate transcription requires target genes that are able to respond to these modest changes in activity. The mechanism by which this is achieved is not yet clear. Moreover in none of the examples of graded signals has this been fully elucidated. Perhaps best understood is the *Drosophila* maternal factor, Dorsal (Stathopoulos and Levine, 2002a; Stathopoulos and Levine, 2002b; Stathopoulos et al., 2002). These studies have defined two general strategies by which graded transcriptional activity can

control differential gene expression. First, the binding affinity of regulatory elements within a gene can control the concentration at which a gene is activated. In the enhancer of *Twist*, which is expressed in the 12-14 most ventral cells, low Dorsal affinity sites were identified; when these low affinity sites were replaced by high affinity sites the expression expanded laterally, detected in the 18-20 most ventral cells (Jiang and Levine, 1993). Consistent with this, high affinity sites were identified in the enhancer of rhomboid which is also regulated by Dorsal but expressed laterally (Ip et al., 1992a; Stathopoulos and Levine, 2002a). These findings suggest that the binding affinities of Dorsal sites specify different limits of expression in response to the nuclear gradient of Dorsal.

A second strategy involves other regulatory regions within a gene which determine the threshold response of a gene to a graded activator or repressor: the presence of a synergistic activator lowers the threshold necessary to activate expression while an inhibitor increases the threshold level required for activation. An example where this mechanism seems to function is the activation of Dorsal target gene *Snail*. The synergistic function of Dorsal with a second transcriptional activator, Twist (Ip et al., 1992b) is important for regulation of *Snail*.

It seems likely that this strategy may be of relevance for the control of Shh responsive genes in the neural tube. In mouse embryos lacking all Gli activity, although the pattern of generation is severely disrupted, many ventral neural tube fates are generated (Bai et al., 2004; Lei et al., 2004), indicating that although Gli proteins are required to provide positional identity, factors other than Gli proteins can induce the expression of ventral genes. Consistent with this SoxB1 proteins have been proposed to act as transactivating factors in neural progenitors (Bylund et al., 2003; Graham et al., 2003), moreover,

signals in addition to Shh, have been proposed to be capable of regulating gene expression characteristic of the ventral neural tube (Diez del Corral et al., 2003; Litingtung and Chiang, 2000; Novitch et al., 2003). Thus it seems plausible that the differential responses of progenitor homeodomain genes are controlled by a combination of Gli binding sites and additional regulatory elements that bind positive and/or negative factors. These additional regulatory elements could determine the threshold at which a gene will respond to Shh and provide a mechanism to 'pre-pattern' the neural tube. Moreover, the previously described cross-repressive interactions between class I and class II homeodomain proteins expressed in complementary domains (Briscoe et al., 2000) would act to ensure a congruent expression profile in progenitors so that individual progenitor cells generate appropriate, distinct neuronal subtypes. A complete molecular understanding of how this operates awaits the identification and characterization of the elements necessary to direct expression of the progenitor homeodomain genes in the neural tube.

4.7 Integrating strength and duration of Shh signalling

The classic model for morphogen action postulates that depending on their distance from a signal source, cells are exposed to different concentrations of the signalling molecule and these different concentrations instruct different cell identities. In addition to the spatial gradient of Shh we endeavoured to test the hypothesis that duration of Shh signalling is involved in providing positional identity. Our data suggest that the duration as well as the strength of Shh signalling influences ventral neural tube patterning. Cells transfected with $Gli3A^{HIGH}$ display markers characteristic of $Gli3A^{MED}$ if assayed at short time points, while increased incubation of embryos transfected with $Gli3A^{MED}$ result in the ectopic induction of markers normally only seen in $Gli3A^{HIGH}$ transfected embryos. Moreover, data from zebrafish embryos exposed to the Smo inhibitor cyclopamine show

that the premature termination of signalling differentially affects the expression of markers characteristics of distinct dorsal-ventral domains of the neural tube; induction of Region A marker Nkx2.2 requires longer exposure to the endogenous Shh signalling (shorter exposure to cyclopamine) compared to Region B marker Olig2 (SV Tsoni, J Briscoe, unpublished data). This suggests that cells respond to Shh by integrating signalling strength over time and not simply responding to the peak signal strength reached. What molecular mechanism could account for these observations? One possible explanation is a mechanism that has been termed the 'sequential cell context' model or 'self-enabling mechanism' (Kang et al., 2003; Pages and Kerridge, 2000). In this model signalling initially regulates an early set of target genes that change the cell context to facilitate the expression of a later set of genes. The cross repressive interactions between class I and II proteins provide obvious candidates for the effect. In the case of Nkx2.2 induction by Shh signalling, a candidate for an early target gene would be repression of Pax6. Gain and loss of function data have provided evidence that Pax6 represses Nkx2.2 expression (Briscoe et al., 2000; Ericson et al., 1997b). Thus, early Shh signalling could downregulate Pax6 providing the appropriate cell context, and then continued Shh signalling would induce the expression of Nkx2.2.

If increased duration of Shh signalling is sufficient to induce increasingly ventral identities what ensures that the entire spinal cord is not ventralized as development and Shh signalling proceeds? An explanation for this may reside in the autoinhibitory characteristic of Shh signalling. Responses to Shh signalling include the induction of Ptc and HIP (Chuang and McMahon, 1999; Pearse et al., 2001), which act as extracellular inhibitors of signalling, it is possible that cell autonomous inhibitors of Shh signalling are also induced by Shh. The induction of inhibitors of Shh signalling acts as an auto-desensitisation mechanism providing a means to limit the ventralization of cells in the

neural tube. This model could also account for the high levels of Shh signalling required for the induction of the most ventral cell types. Thus, in the initial stages of Shh signalling, in which the appropriate cell context is established, partial desensitisation of cells to Shh occurs by the upregulation of genes such as *Ptc*. This would have the consequence that higher levels of Shh signalling are required for the induction of the later, second set of genes. This model also predicts that cells which have the appropriate cell context prior to receiving Shh signall ing would be able to induce target genes at lower thresholds of signal than normally observed. This could explain the increased sensitivity to Shh signalling of, for example, cells of the dorsal midline of the neural tube (roof plate) lacking Pax6 expression (Ruiz i Altaba et al., 1995a). This type of model may provide a means for other signals to influence ventral patterning and suggests that these signals may be integrated at the level of the control of individual genes.

4.8 Effect of Shh signalling manipulation on cell proliferation.

Shh signalling has been associated with cell growth and proliferation in a number of developmental contexts and with certain types of tumorigenesis. Shh upregulates expression of the proto-oncogene *Nmyc* in cultured cerebellar granule neuron precursors and promotes their proliferation (Kenney et al., 2003). In *Drosophila* eye development Hedgehog regulates proliferation by inducing G1/S Cyclins, Cyclin D and Cyclin E (Duman-Scheel et al., 2002). In our experiments where we interfere with Shh signalling in the neural tube, we noticed that blocking or activating the pathway results in outcomes consistent with reduced proliferation (shorter neural tube) or increased proliferation (longer neural tube), respectively. Consistent with this we have detected higher expression of two proliferation markers *Nmyc* and *Cyclin D1* in $Gli3A^{HIGH}$ and $Gli3A^{MED}$ transfected neural cells (B. Cox and J. Briscoe, unpublished data). If the proliferation effect of Gli repressor and activator proteins is confirmed this would lead to the

conclusion that Gli proteins not only mediate Shh patterning but function to control Shh regulated proliferation in the neural tube.

4.9 A proposed model for the function of Gli3-ZF-VP16$_{(4)}$.

As a potentially constitutive Gli activator we assayed a chimeric protein consisting of the DNA binding domain of human Gli3 (zinc fingers) fused to four repeats of the minimal VP16 transactivation domain, Gli3-ZF-VP16$_{(4)}$. Gli3-ZF-VP16$_{(4)}$ works as a very strong transcriptional activator *in vitro*. *In vivo* it causes disruption of the neural tube structure and electroporated cells migrate away from the neural tube. We hypothesize that Gli3-ZF-VP16$_{(4)}$ induces the expression of genes that normally are not Shh/Gli targets. We suggest two possible models that could explain this. One model posits that the zinc finger DNA binding domain of Gli3 is not sufficient, alone, to confer specific binding to Gli promoter targets. As a result Gli3-ZF-VP16$_{(4)}$ binds to and activates genes that are not normally targets of endogenous Gli proteins. The effect of activation of these "irrelevant" genes is dominant to the activation of the normal target genes and cause the phenotype observed. A second model suggests that VP16 recruits a co-activator normally not recruited by Gli proteins. As a result "irrelevant" genes are activated, these are dominant to the activation of the normal target genes and cause the phenotype described above. In this model although the endogenous Gli protein is targeted to genes not normally induced by Shh signalling it does not induce these "irrelevant" targets because the transcriptional cofactors the Gli protein recruits do not activate these genes in the neural tube.

In support of the first model, a family of related transcription factors (*Zic* genes) have been shown to bind common nucleotide sequences with Gli proteins, *in vitro* (Mizugishi et al., 2001). Moreover *Zic1-3* are expressed in the dorsal neural tube and induce neural

crest (Brewster et al., 1998; Nagai et al., 1997). Thus, it is possible that Gli3-ZF-VP16$_{(4)}$ binds to and ectopically induces *Zic* target genes.

To test these possibilities a chimeric protein could be generated containing the DNA binding domain of Gli3, the zinc fingers (ZFs) and a transactivation domain different from the VP16, for example the Gal4. If expression of this construct causes the same effect as Gli3-ZF-VP16$_{(4)}$ then this supports the idea that ZFs alone do not confer specific binding on Gli binding sites. A second approach would be to express a construct containing VP16 domain fused to one of the Gli activators which functions in a specific manner to activate Shh target genes. If the effect of Gli3A-VP16$_{(4)}$ resembles the phenotype caused by the Gli3-ZF-VP16$_{(4)}$ then it is probably the recruitment of an irrelevant co-activator to the Gli binding sites that causes the unexpected phenotype of Gli3-ZF-VP16$_{(4)}$.

4.10 Conclusions

In this study we demonstrated that patterning of the neural tube is sensitive to repression of Gli mediated transcription. Moreover, graded Gli activity is sufficient to emulate patterning effects of Shh gradient generating different ventral neuronal subtypes at different activity levels. The 2-3 fold incremental changes in Shh concentration that generate alternative neuronal subtypes *in vitro* can be mimicked by similar changes in the level of Gli activity *in vivo*, suggesting that the Shh signalling pathway does not substantially amplify signal differences during transduction to the nucleus. In addition to signalling strength the duration of exposure to Shh is an important factor for ventral neural tube patterning, as Shh/Gli activity progressively generates progenitors of more ventral identity.

A number of these features of Shh signalling are shared by other signalling molecules which function as morphogens. First, in the case of Shh, Activin and Dorsal there appears to be a lack of amplification of signal through the transduction pathway, therefore changes in ligand concentration are reflected by similar changes in intracellular signal strength. Second, the signalling pathways of Shh, Activin and Dorsal appear to be linear as both low and high responses are mediated by the same components –receptors, transcription factors. Finally, comparison of Shh and Bicoid suggests that cross repression between the target genes of the graded signal appears to be a common mechanism to refine boundaries of gene expression converting the gradient into thresholds marked by discrete changes in gene expression and cell fates.

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